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Development and Evaluation of Selected Chemoprophylaxis Candidates and a Candidate Live-Attenuated Vaccine for Prevention of Histomoniasis in Turkeys

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Development and Evaluation of Selected Chemoprophylaxis Candidates and a Candidate Live-Attenuated Vaccine for Prevention of Histomoniasis in Turkeys

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ABSTRACT

Histomoniasis, commonly known as blackhead disease, has increased in prevalence due to the regulatory ban of prophylactics and therapeutics within the past 30 years. The objective of this thesis was to evaluate the efficacy of selected dietary chemoprophylaxis candidates as well as an *in vitro* live-attenuated vaccine candidate *Histomonas meleagridis* for prevention of histomoniasis. Chapter one addresses deoxycholic acid and a biliogenic diet intended to endogenously increase production of this secondary bile acid. Deoxycholic acid was effective *in vitro* but failed to prevent histomoniasis when evaluated during the *in vivo* experimental disease trial with turkeys. The biliogenic diet did not reduce disease prevalence. Chapter two addresses dietary inclusion of 0.2% boric acid to prevent disease. The selected concentration of boric acid was unsuccessful in disease prevention. Chapter three addresses the experiments conducted to evaluate select ages, doses, and routes of a candidate live-attenuated vaccine. The live-attenuated vaccine candidate has exhibited slight reduction in histomoniasis severity when administered intracloacally on d14. Although the practicality of this current experimental vaccine administration approach may be limited, further research must be conducted in order to further elucidate conferred immune response and investigate the viability of this vaccine.

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DEDICATION

I would like to dedicate this thesis to my family and friends who have encouraged me throughout this journey. A special dedication belongs to both my tax lady and adopted grandmother who greatly encouraged me throughout my life. They watered the flower of education but sadly did not get to see it bloom.

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I. INTRODUCTION

Histomoniasis (also known as blackhead, infectious enterohepatitis, and histomonosis) is an important disease of poultry, especially turkeys, which can be vectored by the *Heterakis gallinarum* cecal worm, earthworm, or other birds such as chickens (Tyzzer and Fabyan, 1922; van der Heijden et al., 2005; Hess et al., 2015). Caused by the protozoan parasite *Histomonas meleagridis*, initial signs of disease often consist of declined feed consumption, drooping wings, head-tucking, or inactivity (Duffy et al., 2005). Transmission occurs via cloacal drinking which can rapidly transfer pathogens through the cloaca to the bursa of Fabricius or ceca via rhythmic contractions that draw the material inside the vent (Hu et al., 2004; McDougald and Fuller, 2005).

Lack of research within the past 30 years, in addition to the ban of therapeutic and prophylactic compounds, has resulted in lack of methods for controlling this disease (van der Heijden et al., 2005; Hess et al., 2006). There is a need for evaluation of anti-protozoal compounds (chemical and non-chemical) or vaccines for the control of histomoniasis and other protozoal diseases (such as coccidiosis, caused by *Eimeria* spp.). Unfortunately, an alternative to the previously used drugs has not yet been established, because *in vitro* and *in vivo* studies continue to yield variable results against histomoniasis (Thøfner et al., 2012).

Previous immunological research results have been largely unsuccessful, creating doubts for successful vaccine development (Hu and McDougald, 2004). However, in 1963, Joyner treated *H. meleagridis*-infected turkeys with dimetridazole and reported recovered turkeys to be resistant to subsequent infection, suggesting development of protective immunity. Turkeys recovered from *H. meleagridis* infection and subsequently challenged to evaluate immunity have exhibited resistance to characteristic disease symptoms and lesions even when harboring *H.*

meleagridis within the cecae (Cuckler, 1970). Intracloacal administration of *in vitro*-attenuated *H. meleagridis* has resulted in reduced liver and cecal lesions within chickens and turkeys in previous studies (Hess et al., 2008; Liebhart et al., 2013). *In vitro* passaging and subsequent serial passaging of *H. meleagridis* within turkeys and chickens resulted in no reversion to virulence (Sulejmanovic et al., 2013). Furthermore, cross-protection against heterologous virulent isolates was demonstrated by vaccinating with an attenuated clonal strain of *H. meleagridis* developed through prolonged *in vitro* culture methods (Sulejmanovic et al., 2016). These successes encourage continued research in vaccine development as a solution against this disease.

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II. LITERATURE REVIEW

The ban of nitroimidazoles, nitrofurans, and arsenical compounds for prophylaxis and treatment in the last 30 years has led to the lack of preventative options for histomoniasis, a disease primarily affecting turkeys. The following summarizes the disease, chemoprophylaxis compounds focused on the mitigation of this problem, and previous immunization attempts. In addition, an overview of the bile acid pathway and dietary inclusions to increase endogenous production of deoxycholic acid will be summarized, because this compound was selected as an anti-histomonal candidate.

HISTOMONIASIS: A GENERAL OVERVIEW

Histomoniasis (also known as blackhead, infectious enterohepatitis, and histomonosis) is a protozoal disease affecting the gastrointestinal tract of turkeys, chickens, and other gallinaceous birds (van der Heijden et al., 2005; Hess et al., 2015). High mortality occurs in turkeys, whereas less severe damage occurs in chickens and other galliformes (Callait et al., 2002). Considered critically and economically impactful to both turkeys and chickens, histomoniasis is a serious and ongoing concern facing the poultry industry (Duffy et al., 2005; Lotfi et al., 2014).

Histomonas meleagridis is included in the phylum Sarcomastigophora with further taxonomic classification in the order Tritrichomonadida and family Dientamoebidae (Cepicka et al., 2010; Hess and McDougald, 2013). The etiological agent of histomoniasis, *H. meleagridis* penetrates the cecal epithelial lining, replicates, enters the bloodstream and parasitizes the liver (Clarkson, 1963; Hess and McDougald, 2013). Hepatic liver lesions and tissue necrosis are common within diagnosed birds, while individual histomonads can be observed within infected tissues using electron microscopy. Cultivation of *H. meleagridis* has occurred from liver lesions

(Bayon and Bishop, 1937; Bishop, 1938). Overt signs of histomoniasis are often apparent at eleven days post-infection while thickening and reddening of the mucosal layer of the ceca begins within three days of infection (Hess and McDougald, 2013). Although commonly referred to as blackhead within industry and laymen's terms, the clinical sign of cyanosis of the head are neither pathognomonic nor distinctive to histomoniasis because other diseases produce a similar appearance (Sentíes-Cué et al., 2009; Hess and McDougald, 2013). Initial symptoms can include declining feed consumption, drooping wings, unkempt feathers, or inactivity (Duffy et al., 2005). Necrotic typhlitis (necrosis and inflammation of the cecae), hepatitis, sulfuric excreta, and high mortality are characteristic pathological signs (Callait et al., 2002; Hess et al., 2015).

Severe mortality results in turkeys with an estimated annual loss of over two million dollars and mortality approaching 80-100% in an affected flock (Callait et al., 2002; McDougald, 2005; Hess and McDougald, 2013). Although mortality is not as significant in chickens, economic losses are estimated to exceed those of turkeys due to greater frequency of disease and the larger number of flock infections within chickens (Callait et al., 2002). Chicken mortality can be 10-20% with a high morbidity but an outbreak frequently is unnoticed or results in increased condemnations at the broiler processing plant (McDougald, 2005). Mortality is most commonly observed between nine and twelve days post-infection (Hess and McDougald, 2013).

Beginning in the 1960s, research on this organism waned because the introduction of nitroimidazoles, nitrofurans, and arsenical compounds for prophylactic treatment against outbreaks was made available and successfully controlled the disease (van der Heijden et al., 2005; Hess et al., 2006). Concerns over heavy metal retention within meat products from treated poultry resulted in the 2015 regulatory removal of Nitarsone (Histostat), the last remaining FDA-approved drug for treatment of histomoniasis (Regmi et al., 2016). Without viable substitute

treatment options and no new alternatives, producers are now suffering from the losses of broiler breeders, layer pullets, and turkeys (Hu and McDougald, 2004).

Pathogenesis

The trichomonad parasite *H. meleagridis* is capable of existing as either an amoeboid or single-flagellated form, and can be transmitted directly from bird to bird or through intermediate hosts and vectors (Lotfi et al., 2014). Lacking mitochondria, *H. meleagridis* possesses an organelle called the hydrogenosome responsible for anaerobic energy metabolism (McDougald, 2005; Hauck et al., 2010; Hess and McDougald, 2013). These protozoa have a typical median diameter of approximately 10µm, ranging from 6-20µm, and usually exist in amoeboid form but may also exist with a single flagellum (Hess and McDougald, 2013). Recent research has suggested the possible formation of cyst-like stages *in vitro* observed with detailed light and transmission microscopy (Zaragatzki et al., 2010a; b). Isolates can be cultured from infected birds and grown *in vitro*, with long-term cultivation shown to result in a different phenotype, increased growth, and higher tenacity in adverse conditions (Gruber et al., 2017). A larger, nonpathogenic species, *Histomonas wenreichi* or *Parahistomonas wenrichi,* was later discovered and has a typical size of 20-30µm and 3-4 flagella (McDougald, 2005).

Although the life cycle of *H. meleagridis* is not fully understood, it has been characterized as an extracellular parasite that reproduces through binary fission (Tyzzer, 1920; Cuckler, 1970). Direct oral ingestion of histomonads has not recreated the disease reliably in previous research, presumably due to the adverse acidity and mechanical action within the crop and ventriculus (gizzard) of the bird (Hu et al., 2004). Histomonads are delicate in nature and cannot withstand living long periods of time in the environment unless protected by a vector such as the heterakid worm or earthworm (McDougald and Fuller, 2005). The cecal worm

Heterakis gallinarum has been identified as an important vector and reservoir for disease transmission; *H. meleagridis* have been observed within the intestinal wall, larvae, and eggs of this cecal nematode (Hess et al., 2015). *Heterakis gallinarum* appears to provide a protective barrier to transport the infective material through the gastrointestinal tract of poultry, leading to establishment of disease (Tyzzer and Fabyan, 1922; Hu et al., 2004). Ingestion of embryonated heterakid eggs or the adult cecal worms by poultry can result in *H. meleagridis* infection (Hu et al., 2004). Regular earthworms can also ingest histomonad-infected cecal worm eggs, and thereby act as a transport host for the infected heterakid eggs (Lund et al., 1966; Hu et al., 2004). Other protozoa and nematodes have similar transmission methods; the *Enterobius vermicularis* nematode can serve as a vector of *Dientamoeba fragilis,* an intestinal trichomonad parasite primarily of humans, facilitating transmission of disease (Clark et al., 2014).

Rapid transmission by direct contact between infected birds or fecal droppings can occur without an intermediate host or vector if transmission occurs before the fragile histomonads die (Sentíes-Cué et al., 2009). Once a turkey is infected, disease can quickly transmit to others in the flock within two or three days, causing an outbreak (Hess and McDougald, 2013). Interestingly, this method of transmission occurs primarily through the cloacal drinking phenomenon rather than standard fecal-oral or respiratory-respiratory transmission. This cloacal drop or cloacal drinking can rapidly transfer disease through the cloaca to the bursa of Fabricius or ceca via rhythmic contractions that draw the infectious material inside the vent (Hu et al., 2004; McDougald and Fuller, 2005). Infection has been produced with the cloacal injection of infected tissue (liver or cecae) as well as with a suspension of histomonad culture (Berks and Neal, 1952). Recently, oral administration of a clonal *in vitro*-cultured *H. meleagridis* to 1-day-old turkeys followed by 5h feed restriction resulted in histomoniasis mortalities, suggesting a potential oral

infection route if large amounts of contaminated excreta or litter are ingested (Liebhart and Hess, 2009).

To further complicate the understanding of this disease, commensal bacterial flora have been found to be important in disease development (McDougald, 2005). Unfortunately, the cecal environment wherein *H. meleagridis* resides is constantly in a state of flux, making permanent microflora adjustments as a method of controlling this disease difficult (Callait et al., 2002). The nitroimidazole-class drug dimetridazole was previously an effective treatment for histomoniasis; however, the ban of effective prophylactic and therapeutic compounds such as this have resulted in measures focused upon prevention rather than treatment of the disease (Joyner, 1963; Hess and McDougald, 2013; Liebhart et al., 2013). In 2015, the arsenic-based drug nitarsone (Histostat), the last remaining FDA-approved drug for prevention of histomoniasis, was withdrawn from the market due to concerns about inorganic arsenic levels in treated poultry (Regmi et al., 2016). Overall effectiveness is limited, but outbreaks can be reduced with careful animal management practices (Liebhart et al., 2017). The disease is generally more prevalent when birds are housed in environments favoring the coexistence of the cecal nematode *H. gallinarum*, the primary local reservoir for infection (Hess and McDougald, 2013). Chickens often serve as reservoirs for the cecal worm and pose as sources for potential infection; therefore, separate rearing should occur between poultry species to prevent resulting cross-transmission outbreaks (Bayon and Bishop, 1937; McDougald, 2005; Hess and McDougald, 2013).

SELECTED CHEMOPROPHYLAXIS COMPOUNDS

Anti-histomonal drugs, plant-derived compounds, and vaccination attempts have been reviewed by Liebhart et al. (2017). Recent research has focused primarily in the areas of antiprotozoal compounds, antibiotics, vaccines, and plant products with chemical activity (Hess

et al., 2015). Treatment is challenging because protozoa are eukaryotic organisms; selective toxicity is crucial so that the chemoprophylaxis compound is harmful to the parasite without causing irreversible damage to the host. With the increasing world population, finding methods to prevent and treat diseases such as histomoniasis are crucial for the overall well-being of animal husbandry and food production. Additionally, the implications of discovering anti-protozoal compounds that could have beneficial applications towards other protozoal diseases are remarkable and warrant further research for the potential benefit of animals and humans alike. However, identifying alternatives to the previously used drugs has not yet been established, because *in vitro* and *in vivo* studies continue to yield variable results against histomoniasis (Thøfner et al., 2012). The close relationship of *H. meleagridis* to other amoebae and flagellates suggests any anti-protozoal compounds effective against *H. meleagridis* may also have activity against related protozoal species (Hu and McDougald, 2004). If a compound were discovered, this could revolutionize the potential for efficacious treatment of other protozoa such as *Tetratrichomonas* and *Entamoeba* (Hess et al., 2006; Nakada-Tsukui and Nozaki, 2016).

Deoxycholic acid

Deoxycholic acid (**DCA)**, a naturally occurring secondary bile acid, has been shown to reduce severity of *Eimeria maxima* and *Clostridium perfringens* infections in poultry when administered at a concentration of 1.5 g/kg in the diet by reducing intestinal villi damage (Wang et al., 2018). A study in mice resulted in anaerobic bacteria-derived DCA protection against colitis induced by *Campylobacter jejuni* (Sun et al., 2018). Considering these results, the hypothesis that DCA might confer anti-histomonal properties is investigated in the first chapter.

Overview of bile acids. Within the small intestine, the homeostatic control of cholesterol and the absorption of dietary fatty acids are reliant upon biliary lipid secretions that include bile

acids (LeBlanc et al., 1998). Cholic acid (**CA**) and chenodeoxycholic acid (**CDCA**) are the primary bile acids (Stamp and Jenkins, 2008). Cholesterol is an important precursor for bile acids, while bilirubin, heavy metals (such as copper and iron), and urine insoluble lipophilic steroids are constituent components of bile acids (Stamp and Jenkins, 2008; Boyer, 2013). Liver hepatocytes synthesize bile acids, which are water-soluble amphipathic molecules resulting from cholesterol catabolism (Stamp and Jenkins, 2008; Winston and Theriot, 2016). According to Lefebvre et al., (2009), the "classical pathway" for bile acid synthesis uses cholesterol 7α-hydroxylase (**CYP7A1**) and contributes to approximately 75% of bile acid synthesis within the liver. However, the alternative, "acidic pathway" initiated by sterol 27-hydroxylase (**CYP27A1**) is responsible for the remaining 25% of the bile acid synthesis (Lefebvre et al., 2009). The gallbladder functions as the storage organ for bile acids and approximately 5% of these stored bile acids will be fecally excreted (Stamp and Jenkins, 2008). Additionally, cholecystokinin released from the duodenum following a meal initiates gallbladder contraction, stimulating the flow of bile (Ridlon et al., 2006; Lefebvre et al., 2009). A vast canalicular network comprised from apical membranes of hepatocytes and bile duct cholangiocytes results in the bile-secretory unit (Boyer, 2013).

Bile acids contribute to multiple important physiological functions and pathways including: 1) the excretory pathway to clear harmful exogenous substrates that are not readily processed by the kidney; 2) bile salts, which are organic solute components in bile acid that emulsify and facilitate the digestion of fats; 3) the cholesterol elimination pathway; 4) the excretion of IgA, inflammatory cytokines, and innate immune system stimulation; 5) enterohepatic circulation component; and 6) excretion mechanisms for intestinal growth hormones (Boyer, 2013). Bile acids are cytotoxic at high concentrations, and can lead to

carcinogenesis within some visceral organs and tissues (Stamp and Jenkins, 2008; Lefebvre et al., 2009). The hydrophobicity of the bile acids is potentially linked to the toxicity of the molecules, and the increased number of hydroxyl groups is inversely related to toxicity (Stamp and Jenkins, 2008). However, bile acids function as biological detergents, aiding in the production of antimicrobial peptides and contributing to host defense against pathogens (Winston and Theriot, 2016).

Bile acid conjugation. Bile acids are commonly referred to as bile salts, although this may be a more appropriate term when the conjugated bile acids are linked with sodium or potassium salts. Largely impermeable to the intestinal enterocytes, conjugated bile acids are usually restricted to the gastrointestinal lumen due to increased aqueous solubility, ensuring the likelihood of distribution with digesta (Stamp and Jenkins, 2008). The increased levels of bile acids within the lumen initiate micelle formation that contributes to the emulsification and absorption of lipids (Lefebvre et al., 2009). The micelle aids in the diffusion of lipids as they travel the length of the gastrointestinal tract, while also contributing to the reabsorption of bile acids within the distal ileum (Stamp and Jenkins, 2008).

Glycine-conjugated bile acids are the most abundant and represent greater than 70% of bile (Stamp and Jenkins, 2008). The conjugated form is denied entry to epithelial cells thereby protecting the organs and restricting its presence to within the gastrointestinal lumen (Stamp and Jenkins, 2008). Taurine-conjugated bile acids are present in lower abundance but contribute to approximately 20% of bile (Stamp and Jenkins, 2008). Conjugation of CA or CDCA to either glycine or taurine is common; conjugated bile acids are excreted into the lumen of the gastrointestinal tract to facilitate metabolism of fats and fat-soluble vitamins (Van Eldere et al., 1996). Passive reabsorption is prevented due to the stronger acidic nature produced from

conjugation as the bile acids travel through the biliary ducts (Boyer, 2013). The taurine conjugated bile acids have detergent properties and are soluble within the normal acidic stomach environment (Stamp and Jenkins, 2008). This solubility contributes to its potential entry into the gastrointestinal epithelial cells. Not surprisingly, taurine supplementation has be shown to negatively impact intestinal mucosal development through its role in toxic bile acid generation within broiler chickens (Huang et al., 2014).

Free bile acids. Free bile acids are produced via bacterial enzymes that deconjugate primary bile acids within the ileum and the large intestine (Cole and Fuller, 1984). Moreover, bacterial hydroxysteroid dehydrogenases further convert free bile acids into secondary bile acids via a 7α-dehydroxylase reaction. Other enzymatic reactions may occur through 3α- and 7α-hydroxysteroid dehydrogenases as well, but the 7α-dehydroxylation is unique to a few anaerobic species (Winston and Theriot, 2016). Deconjugation reactions within the small intestine and dihydroxylation reactions within the large intestine are the two predominant methods by which secondary bile acids are produced (Winston and Theriot, 2016). Moreover, reconjugation with glycine or taurine can occur, renewing levels of primary and secondary bile acids during enterohepatic recirculation.

Within the lower gastrointestinal tract, the secondary bile acids formed include DCA and lithocholic acids (**LCA**) that are produced from CA and CDCA, respectively (Van Eldere et al., 1996; Ridlon et al., 2006). Many other bile acids may also be formed via bacterial enzymatic reactions and conjugations, but the above mentioned are the more common bile acids (Stamp and Jenkins, 2008). Increased secondary bile acids have been associated with the etiologies of cholesterol gallstone disease and colon carcinogenesis (Winston and Theriot, 2016). Considering this impact against eukaryotic cells, DCA was considered to potentially exhibit toxicity to *H.*

meleagridis. High plasma cholesterol levels, particularly low-density lipoprotein cholesterols, are associated with increased risks of cardiovascular disease (Yang et al., 2012). An inverse relationship appears to exist for CDCA and DCA, meaning that the increasing DCA concentrations are linked with decreasing CDCA (Ridlon et al., 2006). This is understandable in that DCA is formed from the catabolism of CDCA.

Enterohepatic recirculation. The formation of secondary bile acids is important to the enterohepatic circulation that can occur as often as ten times a day. Both primary (CA and CDCA) and secondary (LCA and DCA) bile acids are circulated and reabsorbed through the enterohepatic system (Stamp and Jenkins, 2008). This reabsorption process occurs when DCA and LCA enter the portal veins and circulate to the liver to join the newly synthesized CA and CDCA. Reconjugation to glycine or taurine occurs and these renewed, conjugated bile acids are stored in the gallbladder. The rate-limiting CYP7A1 is suppressed when bile acids are returned to the liver via the enterohepatic circulation, thereby inhibiting additional bile acid synthesis (Stamp and Jenkins, 2008). The actual regulation of synthesis, metabolism, and transportation of bile acids via the enterohepatic system was determined to be self-regulated by the bile acids through activation of the Farnesoid X receptor (**FXR**) (Lefebvre et al., 2009). The FXR is expressed within the liver, gastrointestinal tract, kidneys, and adrenal glands. The recirculated bile acids bind to the FXR in the liver, resulting in negative feedback and downregulation of transcription factors for bile acid synthesis (Stamp and Jenkins, 2008).

If DCA were capable of conferring anti-histomonal properties *in vivo,* use as a commercial feed additive might be more accepted since DCA is naturally occurring and is regenerated during enterohepatic recirculation. Moreover, certain diets have been shown to increase bile acid output that indicates the endogenous processes can be altered by dietary

composition. If DCA effectively mitigates histomoniasis, an alternative to DCA inclusion in diet would be to pursue formulation of a bile-enhancing "biliogenic" diet. This different approach might be more economical than directly supplementing DCA into the feed. The literature considered for formulation of this biliogenic diet is reviewed below.

High protein dietary impacts. Within chickens, the atherogenic impact (formation of fatty plaques in the arteries) of dietary cholesterols and fats was shown to decline with the introduction of higher protein diets (Nishida et al., 1958; Kummerow et al., 1960). Diets high in fat are generally associated with the elevated cholesterol levels that are a precursor to bile acids (Kokatnur et al., 1958b). An inverse linear relationship between protein intake and levels of serum cholesterol was caused in experiments with high protein diets (Kokatnur et al., 1958b). Interestingly, in a study conducted by Kokatnur et al. (1958b), hypercholesteremic birds that were provided adequate or high protein diets showed a rapid decrease in serum cholesterol. Moreover, serum cholesterol values only reached normal levels within hypercholesteremic birds when supplemented with high dietary protein. These observations suggest that increased dietary protein intake might be a more efficient means of reducing overall cholesterol levels in the serum than simply eliminating or decreasing fat consumption (Kokatnur et al., 1958b).

Furthermore, dietary methionine deficiencies were found to increase serum cholesterol (by nearly two-fold as compared to a low protein choline deficient diet), β-lipoprotein levels, and total lipids within the sera of chicks (Kokatnur et al., 1958a; Nishida et al., 1958). These results suggest the artherogenic effects of imbalanced cholesterol, lipid, and methionine when combined with low protein diets (Nishida et al., 1958). Taken together, the apparent connection between dietary protein and artherogenic effects suggest the importance of nutritional management of dietary proteins rather than dietary fats.

The additional role of energy to protein ratio (**E/P ratio**) was considered by Kokatnur et al., (1958a). The E/P ratio was calculated by the following equation:

$$Energy\ protein\ ratio = \frac{total\ calories\ in\ 100\ Gm.}{per\ cent\ protein}$$

Low E/P ratios were associated with significantly decreased serum cholesterol levels, presenting an additional factor to consider when determining dietary effects (Kokatnur et al., 1958a).

Egg-enriched diets. Yang et al. (2012), conducted a study with various egg-enriched diets to evaluate influences upon cholesterol metabolism, among other factors, in rats. Of interest, a diet consisting of 31.25% vacuum freeze-dried egg yolk powder diet (21.78% crude protein) and a diet consisting of 55.56% vacuum freeze-dried whole egg powder (36.09% crude protein) were included in the study. These diets were compared to control groups provided either a standard diet or a 0.75% cholesterol diet. Total bile acid output was higher in all groups as compared to the controls and was particularly significant for the whole egg powder diet. Liver cholesterol levels within both egg-enriched diets were higher than the control, although the 0.75% cholesterol diet exhibited the highest level (Yang et al., 2012). However, this indicates the influence upon hepatic cholesterol levels within egg-enriched diets.

CYP7A1, the rate-limiting enzyme for bile acid synthesis from cholesterol, had increased mRNA expression within the egg-yolk and whole-egg powder enriched diets as compared to both the cholesterol and control diets (Yang et al., 2012). This increased mRNA expression indicates that the egg-enriched diets upregulated the CYP7A1 activity, thereby promoting bile acid synthesis. Moreover, this increased function may have contributed to the elevated output of bile acids within the excreta (Yang et al., 2012). Taken together, the data from this study suggest that egg-enriched diets are consistent with the lowering of cholesterol absorption as well as the increased excretion of bile acids due to increased rate of synthesis.

Lecithin-enriched diets. Lecithin-enriched diets have been shown to increase biliary excretions in both rats and chickens (Lindsay et al., 1969; LeBlanc et al., 1998). In 1969, Lindsay et al. reported an 81% increase in bile acid excretion based upon fecal matter, and a 105% increase based upon bird body weight when chicks were fed a 2.5% lecithin diet. A 0.5% β-sitosterol diet was also found to increase bile excretions, but to a lesser extent than the lecithin diet. Synergistic effects resulted from the combination of the 2.5% lecithin and 0.5% β-sitosterol diets, producing higher bile acid excretion than either diet singly (Lindsay et al., 1969)

LeBlanc et al. (1998) fed rats a 20% lecithin diet that resulted in a 25% increase in bile flow and 61% increase in biliary bile acid output. Interestingly, the lecithin diet also contributed to a proportional change among the bile acid composition, with DCA significantly increasing (LeBlanc et al., 1998). This changed profile could explain why the cholesterol output was also increased.

Considering these factors, Data Chapter 1 evaluates different concentrations of DCA inclusion within a basal diet as well as a diet formulated with the intention of endogenously increasing bile acid synthesis. The above data contributed to our hypothesis that DCA might be effective as a chemoprophylaxis chemical against histomoniasis. However, the complicated nature of histomoniasis serves to further emphasize the importance of pairing *in vivo* studies with *in vitro* experiments to confirm that any chemoprophylaxis activity is conferred within an animal.

Boric acid

Although boron is an essential element to humans, animals, and plants, the NRC (1994) has no recommended level of boron for daily intake in poultry (Eren et al., 2012). Not naturally occurring in elemental form, boron is always bound chemically with oxygen to form borates

(Moore et al., 1997). Boric acid, a boron compound, is used as a litter treatment for the prevention of darkling beetles within the poultry industry, raising concerns that poultry might be harmed by the ingestion of the boric acid litter treatment (Sander et al., 1991; Dufour et al., 1992). Previously, boric acid was shown to be non-toxic when administered orally to 1-day-old chicks at a dose of less than 2g/kg body weight, but levels greater than 3.89 g/kg body weight resulted in high toxicity (Sander et al., 1991). The acute oral mean lethal dose of boric acid in 1-day-old chicks was later determined to be 2.95 ± 0.35 g/kg of body weight, resulting in its classification as a slightly toxic chemical (Sander et al., 1991). Toxic levels of boric acid can result in decreased body weight, increased feed conversion, and abnormal feather growth (Dufour et al., 1992). However, in 1992, Dufour et al. demonstrated that litter treatment with boric acid at a rate of 0.4-0.9kg/9.3m^2 did not significantly increase feed conversion rate or decrease body weight. Previous research has suggested boron may have an important biological role in biochemical mechanisms influencing normal growth and mineral metabolism (Kurtoğlu et al., 2005; Çinar et al., 2015). Beginning at day-of-hatch until d21, dietary concentrations up to 240 ppm (0.024%) boron were not detrimental to broiler performance, although boron levels within breast muscle and liver tissues increased proportionately with increased dietary concentration (Rossi et al., 1993). Moreover, boron supplementation at 20mg/kg in the basal diet had no impact on body weight or feed consumption in chickens; results did not suggest growth-promotion or metabolic mineral regulation (Küçükyilmaz et al., 2017).

Boric acid has antifungal, antiseptic and antiviral properties, and it has been used as an antifungal agent in the treatment of yeast infection (Hernandez-Patlan et al., 2018a). Boric acid has been considered as a prophylactic measure against *Saprolegnia* fungal infections in Atlantic salmon with high hatchability and survival rates following treatment (Ali et al., 2014).

Brittingham and Wilson, (2014) showed that growth rate of *Trichomonas vaginalis,* the protozoan causative agent of trichomoniasis in humans, was reduced with low boric acid concentrations (0.2%) and exhibited lethality to trichomonads at higher concentrations (≤0.4%), independent of environmental acidification. In an *in vitro* gastrointestinal model, boric acid appeared to decrease concentration of *Salmonella* Enteritidis within the intestinal compartment (Hernandez-Patlan et al., 2018a). However, a concentration of 0.1%, boric acid within the basal diet had no significant reduction in *Salmonella* Enteritidis during an *in vivo* study (Hernandez-Patlan et al., 2018b). Dietary supplementation of boron is considered economical in that a 100mg/kg diet was estimated to cost 0.5 US $ per one ton of prepared feed (Bozkurt and Kucukyilmaz, 2015). As bacterial flora are an important factor in development of histomoniasis development, the reduction of bacteria *in vitro* in addition to the antifungal properties exhibited by boric acid contributed to the interest in evaluating this compound for anti-histomonal properties (Hernandez-Patlan et al., 2018a). Considering these experiments, we hypothesized that boric acid might be efficacious against the trichomonad parasite *H. meleagridis* at the selected dietary concentration of 0.2%; this hypothesis is addressed in Data Chapter 2.

VACCINATION

Vaccinations are important for the induction of a host-immune response to protect against disease (Mitra et al., 2018). Previous immunological research results with histomoniasis have largely been unsuccessful; therefore, the likelihood of vaccine development for prevention of this disease has been considered unlikely (Hu and McDougald, 2004). However, turkeys recovered from histomoniasis and subsequently challenged to evaluate immunity have exhibited resistance to characteristic clinical signs and lesions even when harboring *H. meleagridis* within the cecae, suggesting the presence of immune response (Cuckler, 1970). Moreover, *H. meleagridis*-infected

turkeys rescued with dimetridazole were shown to be resistant to subsequent infection, suggesting an acquired protective immunity (Joyner, 1963).

In the early 1900s, intravenous inoculation was found to eventually reproduce histomoniasis when injection was repeated (Tyzzer et al., 1921). Oral administration of fresh subcutaneous, liver, and lung lesions did not reproduce histomoniasis within turkeys (Tyzzer et al., 1921). Tyzzer (1921) stated that turkeys recovered from *H. meleagridis* infection were not able to be re-infected, suggesting immunity. Furthermore, histomoniasis resistance increased with age with only a few mature birds exhibiting clinical signs of infection (Tyzzer et al., 1921). However, turkeys recovered from histomoniasis only exhibited a degree of immunity that appeared transient rather than permanent due to the reappearance of clinically apparent disease several months following recovery from disease challenge (Tyzzer and Fabyan, 1922).

Passive immunization with injection of antiserum from immune into susceptible poultry did not confer protection against histomoniasis upon challenge (Clarkson, 1963; Bleyen et al., 2009). Intramuscular injection of an inactivated clonal *H. meleagridis* also failed to produce effective protection (Hess et al., 2008). These data suggest acquired immune protection is largely cell-mediated rather than humoral (antibody-based). Previous studies with chickens have shown that feed deprivation and an alkaline pH prior to oral challenge were required in order to develop lesions consistent with histomoniasis, suggesting that oral transfer of the parasite should not be overlooked (Cuckler, 1970). Liebhart et al. (2010) demonstrated a protective effect of an *in vitro* attenuated *H. meleagridis* administered orally to 1-day-old turkeys, further suggesting that oral transfer should not be disregarded. A rectally inoculated nonpathogenic strain of *Histomonas* was shown to afford some protection against challenge with pathogenic histomonads three to six weeks post-vaccination, but the effectiveness declined when challenge occurred via infected

heterakid eggs (Lund, 1959). In regards to this outcome, Lund (1959) suggested that the immune barrier was limited to the cecal mucosa, which could be infiltrated by the cecal worms, thus allowing disease development. Reduced albumin and increased γ–globulin are the primary serum protein changes associated with *H. meleagridis* infection (Clarkson, 1966). Recently, vaccination with attenuated histomonads reduced T and B cell subset deviation; mortality in turkeys suffering from histomoniasis was demonstrated to be associated with higher cellular immune response when compared to chickens (Mitra et al., 2017). Co-infection of *H. gallinarum* with *H. meleagridis* resulted in an increased mRNA expression of Th1 cytokine IFN-γ (Schwarz et al., 2011).

Attenuated *H. meleagridis*

Following propagation for two years, an *H. meleagridis* culture originally pathogenic to chickens was found to have lost pathogenicity and induce protection against pathogenic strains when allowed to multiply within the chicken's cecae (Tyzzer, 1932). Early studies by Tyzzer (1934) reported reduced virulence of *H. meleagridis* that was cultivated *in vitro,* although immunization attempts yielded conflicting success. Histomonads passaged *in vitro* more than 1000 times over a period of seven years were shown to be nonpathogenic and considered to have lost efficacy as an immunizing strain capable of protecting against pathogenic *H. meleagridis* strains (Lund and Chute, 1967). Stable attenuation has been shown in a *H. meleagridis* that was passaged 295 times *in vitro* and subsequently serially passaged *in vivo* within turkeys and chickens with no reversion to virulence (Sulejmanovic et al., 2013). More recent studies within chickens and turkeys have shown reduction of liver and cecal lesions following intracloacal administration of clonal *in vitro* attenuated *H. meleagridis* utilized as a vaccine strain (Hess et al., 2008; Liebhart et al., 2013). Nguyen Pham et al. (2013) cloacally inoculated turkeys with a

low-virulence *H. meleagridis* strain that was obtained via serial passage in turkeys and showed induced protection in the face of subsequent challenge by a virulent *H. meleagridis.* Furthermore, an attenuated clonal strain of *H. meleagridis* developed through prolonged *in vitro* culture methods demonstrated a cross-protective capability against heterologous virulent isolates (Sulejmanovic et al., 2016). Pullets vaccinated at 18-week-of-age with an *in vitro*-attenuated, clonal culture exhibited reduced pathology and prevention of a severe drop in egg production as compared to pullets challenged without prior vaccination (Liebhart et al., 2013). Taken together, these data suggest that a protective immune response against histomoniasis may be induced.

Our laboratory obtained a virulent field isolate of *H. meleagridis* that was able to be cultivated and preserved based upon previous methods (van der Heijden et al., 2005; van der Heijden and Landman, 2007). This virulent *H. meleagridis* isolate was propagated for ~80 passages and evaluated as a live-attenuated vaccine candidate. Based upon the rapid *in vitro* growth and perceived virulence reduction within turkeys, we believed that it may be a worthy vaccine candidate, a topic that will be further explored in Data Chapter 3 within a disease challenge study.

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III. DATA CHAPTER 1

Evaluation of deoxycholic acid as a prophylactic treatment to prevent histomoniasis in turkeys

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ABSTRACT

Deoxycholic acid (DCA) is a naturally occurring secondary bile acid, originating from intestinal bacterial metabolic conversion of cholate, a primary bile acid. DCA has been shown to have anti-histomonal properties *in vitro*, leading to our hypothesis that DCA inclusion within the feed may prevent histomoniasis in turkeys. Selected concentrations of DCA within a basal starter diet were evaluated for effects on body weight gain (BWG), lesions, and mortality of *H. meleagridis*-challenged turkeys. Treatments consisted of Negative Control, 0.25% DCA diet, 0.5% DCA diet, 1% DCA diet, or Wild-Type (WT) Positive Control. The basal turkey starter diet was fed to all groups until d7, at which time DCA diets were administered to respective groups. Via intracloacal inoculation, $2x10^5$ WT *H. meleagridis* cells/turkey were administered on d14, and lesions were evaluated d13 post-challenge. Pre-challenged d0-14 BWG in the 0.25% DCA group was higher ($p \leq 0.05$) than the 1% DCA group. There were no significant differences in pre-challenge d0-14 BWG between any of the other groups. No significant differences in mortalities from histomoniasis occurred in DCA treatment groups as compared to the WT Positive Control. No lesions or mortalities characteristics of histomoniasis were observed at any time in the Negative Control poults. Presence of classic Histomoniasis-related liver lesions was statistically higher in the 0.5% DCA diet as compared to the WT Positive Control. Utilizing the same controls and experimental timeline, an additional group was included to evaluate a biliogenic diet that was formulated to encourage endogenous bile acid production. The biliogenic diet had no statistical impact on pre-challenge d0-14 BWG, but this diet did not reduce mortality or lesions related to histomoniasis. Taken together, these data suggest DCA inclusion within the feed at these concentrations and under these experimental conditions does not prevent Histomoniasis. Although DCA treatment reduced *H. meleagridis* cells *in vitro*, the *in vivo* trial

resulted in no reduction of mortalities or lesion presence from histomoniasis within the DCA diets as compared to the WT Positive Control.

Key Words: blackhead, deoxycholic acid, histomoniasis, *Histomonas meleagridis*, turkey

INTRODUCTION

Histomoniasis, also known as blackhead, is an important disease particularly affecting turkeys in addition to other gallinaceous birds (van der Heijden et al., 2005; Hess et al., 2015). Caused by the protozoan parasite *Histomonas meleagridis*, mortality can approach 80-100% of the flock with significant economic damage incurred (Callait et al., 2002; McDougald, 2005; Hess and McDougald, 2013). Nitroimidazoles were previously an effective treatment for histomoniasis; however, regulatory action resulted in the removal of effective prophylactic and therapeutic compounds such as this without any alternatives introduced for disease treatment (Joyner, 1963; Hess and McDougald, 2013; Liebhart et al., 2013).

Deoxycholic acid (**DCA**) is a naturally occurring secondary bile acid produced through intestinal bacterial metabolic conversion of cholate (Van Eldere et al., 1996; Ridlon et al., 2006). DCA has been shown to reduce severity of *Eimeria maxima* and *Clostridium perfringens* poultry infections when administered in a dietary concentration of 1.5 g/kg with an associated reduction of damaged intestinal villi (Wang et al., 2018). A study in mice demonstrated that anaerobic bacteria-derived DCA protected against colitis induced by *Campylobacter jejuni* (Sun et al., 2018). The primary purpose of the present study was to evaluate DCA as a chemoprophylaxis candidate against histomoniasis. In addition, dietary composition has been shown to influence endogenous production of bile acids within the host (Yang et al., 2012). Therefore, a biliogenic diet was formulated to encourage endogenous bile acid formation in the turkey with the hypothesis that severity of histomoniasis would be reduced.

MATERIALS AND METHODS

In vitro *Assessment of Deoxycholic Acid*

Three *in vitro* assays were completed to evaluate selected concentrations of high purity DCA sodium salt (VWR International LLC, USA). Wild-type (**WT**), virulent *H. meleagridis* were added at a ratio of 100µL histomonads: 50µL DCA treatment into a 96-well, sterile microtiter plate. Each treatment was performed in pentaplicate. Incubation occurred at 40°C with wells capped and parafilm utilized to maintain anaerobic conditions. Following incubation, viable histomonads were enumerated by trypan blue 0.4% vital dye exclusion using a hemocytometer, and cell counts were expressed as viable histomonads/mL.

In Assay 1, a concentration of $2.01x10^6$ histomonads/mL of WT *H. meleagridis* was added according to the method above. Treatments included sterile phosphate-buffered saline (PBS) as a negative control or selected final DCA concentrations of 0.4, 2, and 4mM. The plate was incubated 7-8.5 hours before viable histomonads were enumerated as described above. In Assay 2, a concentration of $6.88x10^5$ histomonads/mL was used and treatments included either PBS control or 0.5, 1, 2, or 4mM DCA concentrations. The plate was incubated 6-8 hours before viable histomonads were enumerated. In Assay 3, a concentration of $6.35x10^5$ histomonads/mL was added and treatments included either PBS or DCA concentrations of 0.5, 1, or 2mM. The plate was incubated and viable histomonads were enumerated at two time periods of 4-6 hours and 27-29 hours.

Animal Source and Diet

A total of 140 day-of-hatch female turkey poults were obtained from a local commercial hatchery. Poults were neck-tagged individually and randomly allocated to floor pens at the University of Arkansas Poultry Health Laboratory. All animal handling procedures were in

compliance with the Institutional Animal Care and Use Committee (IACUC protocol #18113) of the University of Arkansas. A corn-soy-based starter feed that met or exceeded nutrient requirements for poultry (NRC, 1994) and water were provided *ad libitum*. Early poult mortalities unrelated to histomoniasis were recorded and altered group numbers reported in the experiment.

DCA diet. On d7, DCA was included in the diet at selected concentrations of either 0.25, 0.5, or 1%. Treatments consisted of Negative Control (n=59), 0.25% DCA diet (n=20), 0.5% DCA diet (n=20), 1% DCA diet (n=20), and WT Positive Control (n=20). Turkeys remained on treatment diets for the remainder of the experiment.

Biliogenic diet. Utilizing the same positive and negative controls as above, a Biliogenic diet treatment group (n=20) consisting of 20% whole egg powder (Heartland Supply Co., USA) inclusion within a basal turkey starter was evaluated. This treatment's objective purpose was to physiologically upregulate natural bile acid synthesis to potentially increase endogenous DCA production within the turkey (**Table 1**). This group was subjected to the same experimental timeline and evaluation methods as the DCA treatment groups mentioned previously (**Figure 1**).

Histomonas meleagridis *Challenge*

To initiate disease challenge, all poults other than the Negative Control received a total dose of $2x10^5$ WT, virulent *H. meleagridis* cells/poult administered intracloacally with an animal gavage needle on d14. Inoculation occurred twice (at half total dosage) with 1h between inoculations to ensure each bird received an infectious dose.

Lesion Scores and Body Weight Gain

All poults were weighed individually on d0 and d14 for calculation of pre-challenge body weight gain (**BWG**). Presence liver and cecal lesions associated with histomoniasis was recorded

from all mortalities following challenge. On d13, all remaining poults were necropsied for the presence or absence of liver and cecal lesions typical of histomoniasis.

Statistical Analysis

In vitro data were computed using JMP Pro 14 software. Significant differences between viable histomonads/mL in treatment groups were determined using ANOVA, and means were further separated using Tukey's multiple comparison post hoc test with values of $p \leq 0.05$ considered significant. Pre-challenged BWG data were also analyzed using JMP Pro 14 software, with significant differences between BWG in treatment groups determined using ANOVA. Where applicable, means were further separated using Tukey's multiple comparison post hoc test with values of $p \leq 0.05$ considered significant. Mortalities and lesion presence related to histomoniasis were compared against the WT Positive Control using chi-square test with a difference of $p \leq 0.05$ considered significant.

RESULTS

In vitro *Cell Viability Assays*

In assay 1, mean viable histomonads/mL (Log10) for PBS, 0.4mM DCA, 2mM DCA and 4mM DCA treatments following 7-8.5h incubation were 6.22, 6.25, 0.00, and 0.00, respectively (**Table 2**). The treatments of 2mM and 4mM DCA significantly reduced the concentration of viable histomonads as compared to either the PBS negative control or the 0.4mM DCA.

In assay 2, mean viable histomonads/mL (Log10) for PBS, 0.5mM DCA, 1mM DCA, 2mM DCA, and 4mM DCA treatments following 6-8h incubation were 6.11, 6.12, 4.71, 0.00, and 0.00, respectively. The treatments of 1mM, 2mM, and 4mM DCA significantly reduced the concentration of viable histomonads as compared to either the PBS or the 0.5mM DCA.

In assay 3, mean viable histomonads/mL (Log10) for PBS, 0.5mM DCA, 1mM DCA, and 2mM DCA following 4-6h incubation were 6.30, 6.31, 6.16, and 1.34, respectively. The 1mM DCA treatment significantly reduced the viable histomonads as compared to PBS or 0.5mM DCA. The 2mM DCA had lower viable histomonads as compared to all other treatments. Following 27-29h incubation, histomonads from assay 3 were enumerated with mean counts of 6.48, 6.18, 4.46, and 0.00, respectively. The 0.5mM DCA treatment had lower viable histomonad counts as compared to the PBS. The 1mM and 2mM DCA significantly reduced viable histomonads as compared to either the PBS or 0.5mM DCA treatments.

Pre-challenge BWG from d0-14

The selected dietary concentrations of DCA had no statistically negative impact on d0-14 pre-challenged BWG as compared to the basal control diet. The 0.25% DCA diet had higher ($p \leq 0.05$) pre-challenge BWG as compared to the 1% DCA diet (**Table 3**). The 1% DCA diet was lower at pre-challenge BWG than the Negative Control but was not statistically different. No differences were observed in pre-challenge BWG between any of the other DCA treatments.

Histomoniasis Infection Response and Lesions

Differences in mortalities associated with histomoniasis were not significant between any of the DCA treatments as compared to the WT Positive Control (**Figure 2**). Presence liver and cecal lesions associated with histomoniasis was significantly higher in the 0.5% DCA diet as compared to the WT Positive Control (**Figure 3**). No classical lesions associated with histomoniasis were observed at any time in the Negative Control.

Biliogenic Dietary Impact on Histomoniasis

The biliogenic diet did not reduce histomoniasis. No statistical difference was detected in the d0-14 pre-challenge BWG (**Figure 4A**). Mortalities and lesions related to histomoniasis were

not reduced in the biliogenic diet group as compared to the WT Positive Control (**Figures 4B** and **4C**, respectively).

DISCUSSION

In vitro testing of DCA effectively reduced the viability of *H. meleagridis*; viable histomonad counts decreased with increased concentration of DCA. However, dietary inclusion at the selected concentrations neither prevented nor mitigated the disease in *H. meleagridis*-challenged turkeys. Although not statistically different, BWG during the pre-challenge phase decreased with increased dietary DCA as compared to the basal diet. This could indicate that the maximum acceptable concentration of DCA inclusion was approached, and higher DCA inclusion rates may not be useful if any further research is conducted. Again, although not statistically significant, the 0.25% and 0.5% DCA groups had higher mortalities as compared to the WT Positive Control. The higher lesion presence within the 0.5% DCA group as compared to the WT Positive Control further contributes to the conclusion from this study that DCA was not effective against histomoniasis under these experimental conditions. A higher inclusion rate of DCA would be discouraged unless further evaluation was completed to evaluate physiological impact. In addition, a higher concentration of DCA would likely be economically unfeasible. Mortalities and lesions characteristic of histomoniasis were not reduced by the biliogenic diet. Similarly to the DCA diets, the biliogenic diet numerically (not statistically) reduced pre-challenge BWG as compared to the basal diet. Mortalities and lesions were not statistically different, although the biliogenic diet marginally increased the rates of both. If future studies were to be conducted, it might be beneficial to include measurement of feed intake to evaluate whether the poults were ingesting adequate amounts of feed compared to the basal diet.

According to Lefebvre et al. (2009), the "classical pathway" for bile acid synthesis contains the cholesterol 7α-hydroxylase (CYP7A1) and contributes to approximately 75% of bile acid synthesis within the liver. Cholesterol, an important precursor for bile acids, has recently been shown to enhance *H. meleagridis* growth *in vitro* (Gruber et al., 2018). Based on this recent finding by Gruber et al. (2018), the biliogenic diet formulation, which included 20% whole egg powder, may have increased normal cholesterol levels and inadvertently aided the parasite. Both lecithin-enriched and egg-enriched diets have been associated with increased total bile acid output (Lindsay et al., 1969; LeBlanc et al., 1998; Yang et al., 2012), but no measurements of bile acids were evaluated within the present study. Although DCA displayed significant reduction of histomonads *in vitro*, the ineffectiveness to prevent histomoniasis *in vivo* is consistent with the variable results of other candidates evaluated against this disease. Extracts of the medicinal herb *Artemisia annua* as well as artemisinin (a main active compound) previously exhibited anti-histomonal properties *in vitro* but failed to prevent infection within experimental challenged turkeys and chickens (Thøfner et al., 2012). The same *H. meleagridis* culture was utilized by Thøfner et al. (2012), but *in vitro* susceptibility did not translate to *in vivo* results. These findings are similar to the DCA results, further emphasizing the importance of incorporating *in vivo* evaluation of anti-histomonal chemoprophylaxis compounds rather than relying only upon positive *in vitro* data. Further confounding this already complicated disease, the cecal environment wherein *H. meleagridis* resides is constantly in a state of flux, demonstrating the difficulties in controlling histomoniasis. Bacterial flora contribute an important role in disease development; therefore, influence of chemoprophylaxis candidates on the gastrointestinal environment as well as the parasite should be considered (Callait et al., 2002; McDougald, 2005). In conclusion, DCA and the formulated biliogenic diet were evaluated for

the first known time against histomoniasis but none of the dietary treatments were effective in the mitigation of disease.

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TABLES

Table 1. Ingredient composition of the biliogenic diet for induction of endogenous bile acids.

Item[1]	% as-fed
Corn	45.7
Soybean meal	28.2
Spray-dried egg powder[2]	20.0
Dicalcium phosphate	2.75
Limestone	1.52
Poultry Fat	1.00
L-lysine HCl	0.23
Salt	0.20
DL-methionine	0.15
CT Tky Starter VTM	0.15
Choline chloride (60%)	0.07
L-threonine	0.01

[1]68mg of biotin was added to the premix
[2]Spray-dried egg powder, Heartland Supply Co., Fayetteville, AR.

Table 2. *In vitro* viability assays evaluating selected concentrations of deoxycholic acid for anti-histomonal properties.[1,2]

ASSAY 1	Viable Cells/mL (Log_{10})	
Treatment	After 7-8.5 hr. incubation	
PBS	6.22 ± 0.02^{a}	
0.4 mM DCA	6.25 ± 0.03^{a}	
2 mM DCA	0.00 ± 0.00^{b}	
4 mM DCA	0.00 ± 0.00^{b}	
ASSAY 2	Viable Cells/mL (Log_{10})	
Treatment	After 6-8 hr. incubation	
PBS	6.11 ± 0.02^{a}	
0.5 mM DCA	6.12 ± 0.05^{a}	
1 mM DCA	4.71 ± 0.25^{b}	
2 mM DCA	0.00 ± 0.00^{b}	
4 mM DCA	0.00 ± 0.00^{b}	
ASSAY 3	Viable Cells/mL (Log_{10})	
Treatment	After 4-6hr. Incubation	After 27-29 hr. incubation
PBS	6.30 ± 0.05^{a}	6.48 ± 0.01^{a}
0.5 mM DCA	6.31 ± 0.03^{a}	6.18 ± 0.07^{b}
1 mM DCA	6.16 ± 0.04^{b}	4.46 ± 1.25^{c}
2 mM DCA	1.34 ± 0.92^{c}	0.00 ± 0.00^{c}

[a-c]Data are expressed as the Mean ± SEM, n=5 samples. Statistical evaluation using ANOVA followed by Tukey's multiple post-hoc test. Means with no common superscript differ significantly ($p \leq 0.05$).
[1]Assay 1-3 began with concentrations of $2.01x10^6$, $6.88x10^5$, and $6.35x10^5$ histomonads/mL of the wild-type *H. meleagridis*, respectively, added at a ratio of 100µL cells: 50µL treatment and incubated under anaerobic conditions at 40°C.
[2]DCA=Deoxycholic acid

Table 3. Effect of dietary inclusion of selected concentrations of deoxycholic acid on d0-14 BWG during pre-challenge phase.

Treatment	d0-14 Pre-Challenge BWG (g)[1]
Wild-Type Positive Control d14	270 ± 9.36^{ab}
0.25% DCA	289 ± 14.0^{a}
0.5% DCA	257 ± 13.1^{ab}
1% DCA	242 ± 12.4^{b}
Negative Control	265 ± 6.34^{ab}

[a,b]Means ± SEM with no common superscript differ significantly ($p \leq 0.05$).
[1]Statistical evaluation using ANOVA followed by Tukey's multiple post-hoc test.
[2]DCA=Deoxycholic Acid.

FIGURES

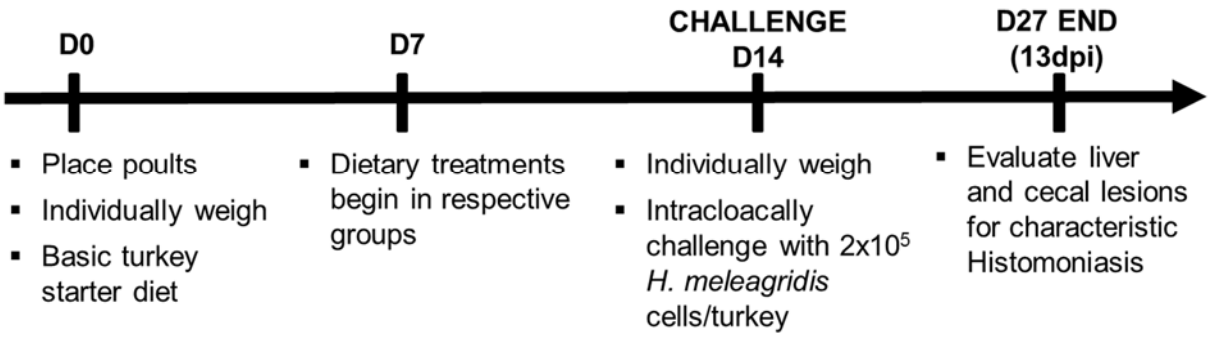

Figure 1. *In vivo* trial experiment timeline.

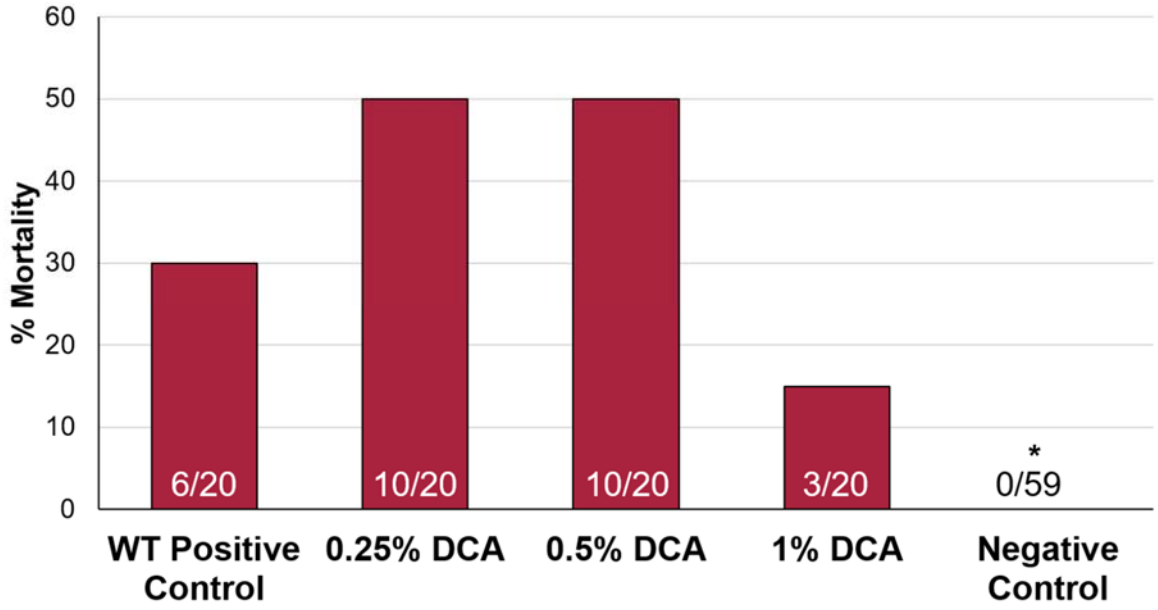

Figure 2. Percentage post-challenge cumulative mortality related to histomoniasis. Statistical difference indicated by "*" to indicate $p \leq 0.05$ as compared to the Wild-Type (WT) Positive Control. No differences were detected when DCA was compared to the WT Positive Control.

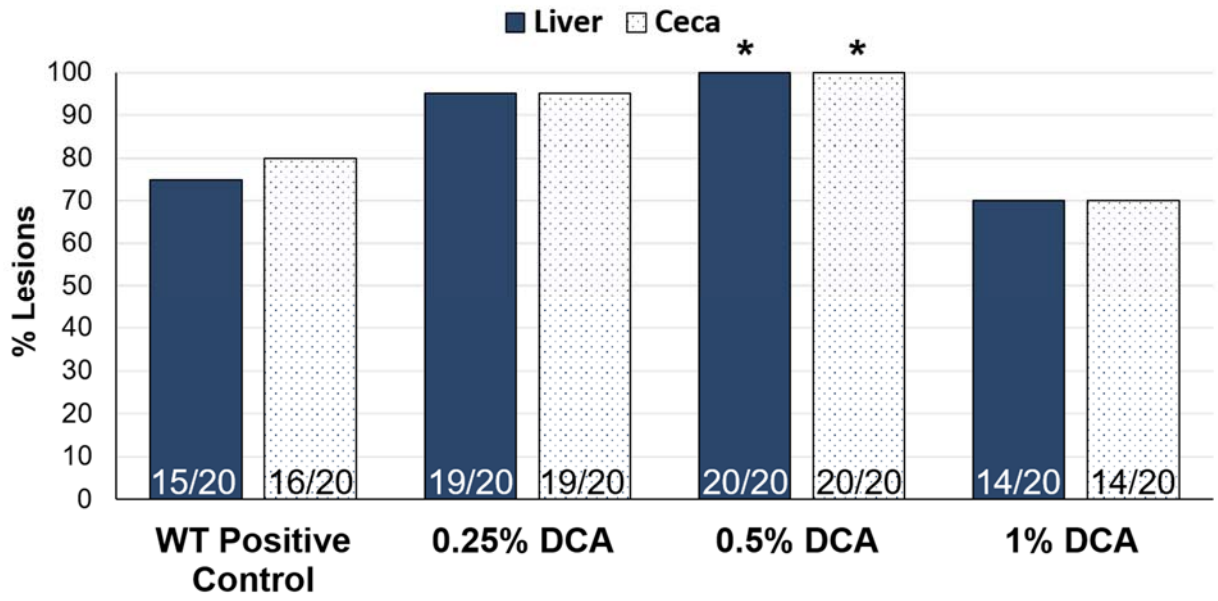

Figure 3. Percentage cumulative liver and cecal lesions associated with histomoniasis beginning from 9d post-challenge until d13 experiment termination. Lesions were determined based on the presence or absence of classic histomoniasis that is characterized by target-like liver lesions and cecal cores. Statistical differences indicated by "*" to indicate $p \leq 0.05$ as compared to the Wild-Type (WT) Positive Control. No lesions or mortalities associated with histomoniasis were detected at any time within the Negative Control.

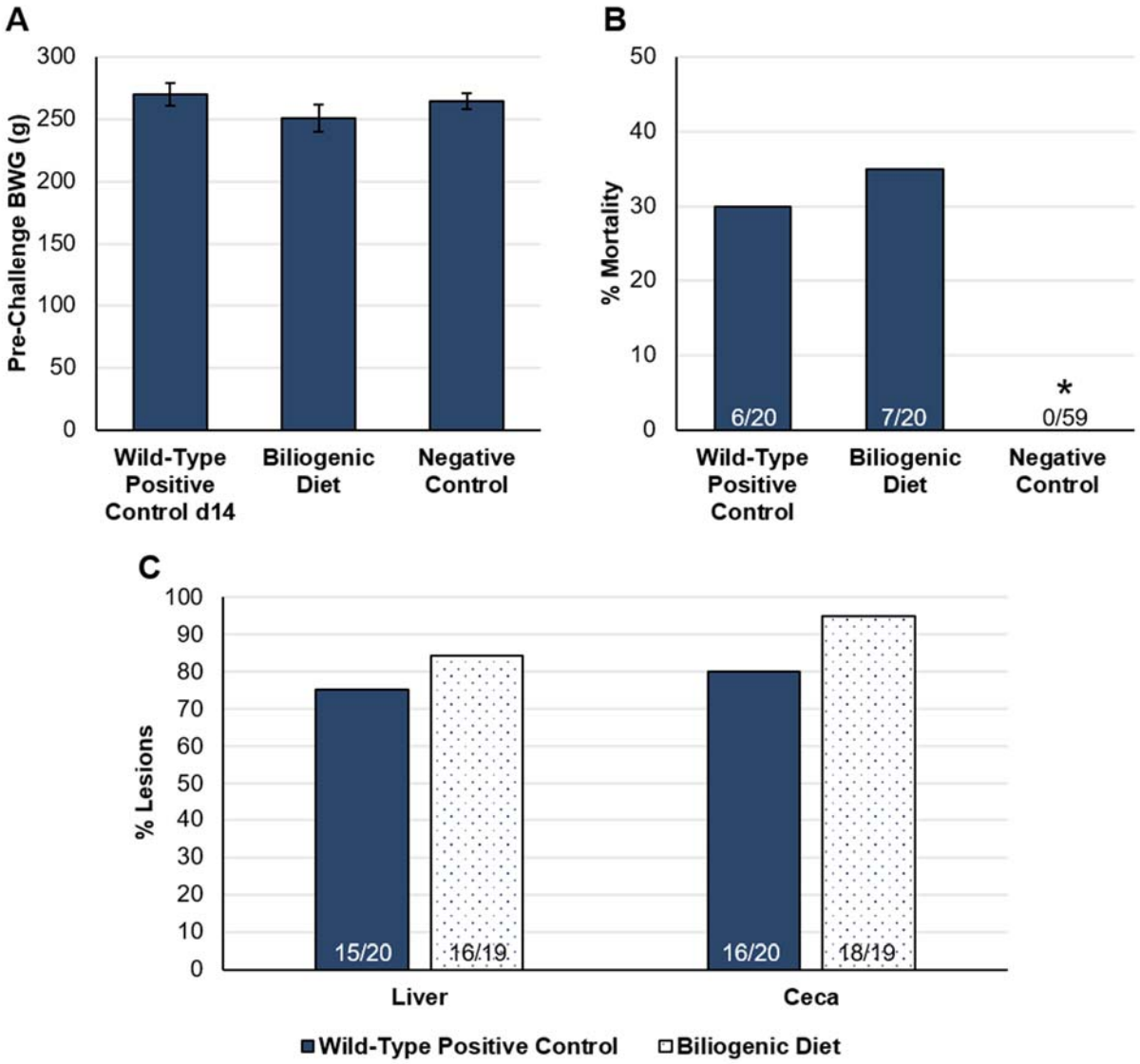

Figure 4. A) Biliogenic dietary effect on d0-14 BWG during pre-challenge phase. No significant differences were found between BWG. **B)** Post-challenge cumulative mortality associated with histomoniasis. Statistical difference indicated by "*" to indicate $p \leq 0.05$ as compared to Wild-Type (WT) Positive Control. **C)** Percentage cumulative liver and cecal lesions associated with histomoniasis beginning from d6 post-challenge until d13 experiment termination. No significant differences in lesions associated with histomoniasis were found between the biliogenic diet group and WT Positive Control following WT *H. meleagridis*-challenge.

IV. DATA CHAPTER 2

Evaluation of boric acid as a chemoprophylaxis candidate to prevent histomoniasis

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ABSTRACT

Histomoniasis, caused by the protozoan parasite *Histomonas meleagridis*, is a disease to which turkeys are especially susceptible. Currently, no chemoprophylaxis compounds are available to mitigate this disease. Boric acid exhibits antifungal, antiseptic and antiviral properties, and it has been used in the treatment of yeast infections. Based upon these characteristics, an experiment was conducted to evaluate whether boric acid might be efficacious against *H. meleagridis*. Treatments consisted of Negative Control, 0.2% Boric Acid diet, and Wild-Type Positive (WT) Challenged Control. The 0.2% Boric Acid diet was administered to the respective group beginning on day-of-hatch. On d21, challenge with $2x10^5$ *H. meleagridis* cells/turkey occurred intracloacally, and lesions were evaluated on d14 post-challenge. Individual body weights were recorded on d0, 21, and 35 to calculate the pre-challenge and post-challenge body weight gain (BWG). The 0.2% Boric Acid group resulted in lower pre-challenge d0-21 BWG ($p \leq 0.05$) than the Negative Control. Post-challenge d21-35 BWG was not statistically lower ($p=0.0567$) than the wild-type positive control. No differences were detected in mortalities associated with histomoniasis between the 0.2% Boric Acid group and the WT Positive Control. Liver and cecal lesions were not statistically different between the 0.2% Boric Acid group and the WT Positive Control. Taken together, these data suggest that boric acid was not efficacious in the prevention or reduction of disease severity when provided at this dietary concentration under these experimental conditions.

Key words: blackhead, boric acid, boron, histomoniasis, *Histomonas meleagridis*

INTRODUCTION

Histomoniasis, also commonly known as blackhead, is a disease of turkeys associated with high mortality (Callait et al., 2002). Considered critically and economically impactful to both turkeys and chickens, histomoniasis is a serious concern facing the poultry industry (Duffy et al., 2005; Lotfi et al., 2014). *Histomonas meleagridis,* the etiological agent of histomoniasis, penetrates the cecal epithelial lining, replicates, enters the bloodstream, and parasitizes the liver (Clarkson, 1963; Hess and McDougald, 2013). Research on this organism waned in the 1960s following the introduction of nitroimidazoles, nitrofurans, and arsenical compounds for prophylaxis and treatment of outbreaks; compounds have since been banned due to regulatory action (van der Heijden et al., 2005; Hess et al., 2006). In 2015, the arsenic-based drug nitarsone (Histostat), the last remaining FDA-approved drug for prevention of histomoniasis, was withdrawn from the market due to concerns about inorganic arsenic residues in treated poultry (Regmi et al., 2016). Unfortunately, no alternative to the previously used drugs has been identified; *in vitro* and *in vivo* studies continue to yield variable results against histomoniasis (Thøfner et al., 2012).

Boron is an essential element to humans, animals, and plants (Eren et al., 2012). The NRC (1994) has no recommended level of boron for daily intake in poultry. Within the poultry industry, application of boric acid, a boron compound, to the litter is used in the prevention of darkling beetles (Sander et al., 1991; Dufour et al., 1992). In 1992, Dufour et al. showed that boric acid litter treatment at a rate of 0.4-0.9kg/9.3m^2 did not significantly increase feed conversion or decrease body weight. Previous studies have suggested the important biological role that boron may have on the biochemical mechanisms influencing mineral metabolism and normal growth (Kurtoğlu et al., 2005; Çinar et al., 2015). Dietary supplementation of boron is

considered economical in that a 100mg/kg diet was estimated to cost 0.5 USD per ton of prepared feed (Bozkurt and Kucukyilmaz, 2015). Beginning at day-of-hatch until 21d, up to 240 ppm (0.024%) boron within the diet was not detrimental to broiler performance, although boron levels within breast muscle and liver tissues increased in proportion boron dietary concentration (Rossi et al., 1993). Boron (20mg/kg) supplementation in a basal diet had no impact on body weight or feed consumption in chickens; results did not suggest growth-promotion or metabolic mineral regulation (Küçükyilmaz et al., 2017). However, the acute oral mean lethal dose of boric acid in 1-day-old chicks was determined to be 2.95 ± 0.35 g/kg of body weight, resulting in the classification of boron as a slightly toxic chemical (Sander et al., 1991).

Containing antifungal, antiseptic and antiviral properties, boric acid has also been used to treat yeast infections (Hernandez-Patlan et al., 2018a). Within an *in vitro* model, boric acid decreased concentration of *Salmonella* Enteritidis within the intestinal compartment (Hernandez-Patlan et al., 2018a). However, during an *in vivo* study, a concentration of 0.1% boric acid within the basal diet had no significant reduction in *Salmonella* Enteritidis (Hernandez-Patlan et al., 2018b). The growth rate of *Trichomonas vaginalis,* protozoan causative agent of trichomoniasis in humans, was reduced with low boric acid concentrations (0.2%) and exhibited lethality to trichomonads at higher concentrations (≤0.4%), independent of environmental acidification (Brittingham and Wilson, 2014). Bacterial flora are important in development of histomoniasis, contributing to the interest in boric acid as a potential chemoprophylactic compound against the disease. Considering these experiments, the antifungal properties, and the potential cost-effectiveness, we hypothesized that boric acid might be efficacious against the trichomonad *H. meleagridis* at the selected dietary concentration of 0.2%.

MATERIALS AND METHODS

Animal Source and Diet

A total of 120 day-of-hatch female turkey poults were obtained from a local commercial hatchery. Poults were neck-tagged individually and randomly allocated to floor pens at the University of Arkansas Poultry Health Laboratory. Early poult mortalities unrelated to histomoniasis were recorded and the altered group numbers are reported in the experiment. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC protocol #18113) of the University of Arkansas. A corn-soy-based starter feed that met or exceeded nutrient requirements of poultry (NRC, 1994) and water were provided *ad libitum*. Treatments consisted of Negative Control (n=34), 0.2% Boric Acid diet (n=27), and Wild-Type (**WT**) Positive Challenged Control (n=20). Beginning on day-of-hatch, boric acid (Sigma-Aldrich, St. Louis, MO) was incorporated into the basal diet at a concentration of 0.2% for the treatment group receiving the boric acid diet.

Histomonas meleagridis

On d21, all poults other than the Negative Control received a total dosage of $2x10^5$ WT, virulent *H. meleagridis* cells/turkey administered intracloacally with an animal gavage needle. Inoculation occurred twice with a 1h period between each inoculation.

Lesion Scores and Body Weight Gain

All poults were individually weighed on d0, 21, and 35 for calculation of pre-challenge and post-challenge body weight gain (**BWG**). Liver and cecal lesions associated with histomoniasis were tabulated from all mortalities. On d14 post-challenge, all remaining poults were necropsied to evaluate liver and cecal lesions. Classical lesions associated with histomoniasis were separately recorded on a scale of 0-3, with 3 being the most severe.

Statistical Analysis

BWG data were analyzed using JMP Pro 14 software, with significant differences between treatment groups determined using a Student's t-test with $p \leq 0.05$ considered significant. Differences in mortalities associated with histomoniasis were analyzed using chi square test. Lesion score data were analyzed using the Proc Mixed Procedure in SAS 9.4 software with significance between mean lesion score values considered at $p \leq 0.05$ in comparison to the WT Positive Control.

RESULTS

Pre-challenge BWG from d0-21 was significantly lower in poults fed the 0.2% Boric Acid diet as compared to poults fed the basal diet in the Negative Control group (Figure 1A). Post-challenge BWG from d21-35 in the 0.2% Boric Acid group was numerically lower than the WT Positive Control but not statistically different with $p=0.0567$ (Figure 1B). No differences were detected in mortalities associated with histomoniasis in the 0.2% Boric Acid group as compared to the WT Positive Control (Figure 1C). Moreover, neither liver nor cecal lesions were reduced in the 0.2% Boric Acid group as compared to the WT Positive Control following *H. meleagridis*-challenge (Figures 1D and 1E, respectively).

DISCUSSION

At this selected dietary concentration of boric acid, the significantly lower pre-challenged BWG suggests that the level of boric acid should not exceed 0.2% in the diet fed to turkeys. Lowered body weight gain is consistent with previous literature indicating that toxic levels of boric acid can induce increased feed conversion and decreased body weight (Dufour et al., 1992). Boric acid previously resulted in high toxicity when orally administered to 1-day-old chicks at levels greater than 3.89 g/kg body weight (Sander et al., 1991). Efficacy of alternative

chemoprophylaxis compounds against related protozoa is suggested due to the close relationship of *H. meleagridis* to other amoebae and flagellates (Hu and McDougald, 2004). However, selective toxicity against protozoa is crucial to ensure that the chemoprophylaxis compound is harmful to the parasite without causing irreversible damage to the host. The early mortalities excluded from the data set could potentially have resulted from boric acid toxicity, but tissue levels and lesions other than those characteristic to histomoniasis were not considered within this experimental design. Overall, these data suggest that boric acid at this selected dietary concentration and under these experimental conditions is not effective in the mitigation of histomoniasis. If continued, future research should consider boric acid concentrations less than 0.2% within the diet.

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FIGURES

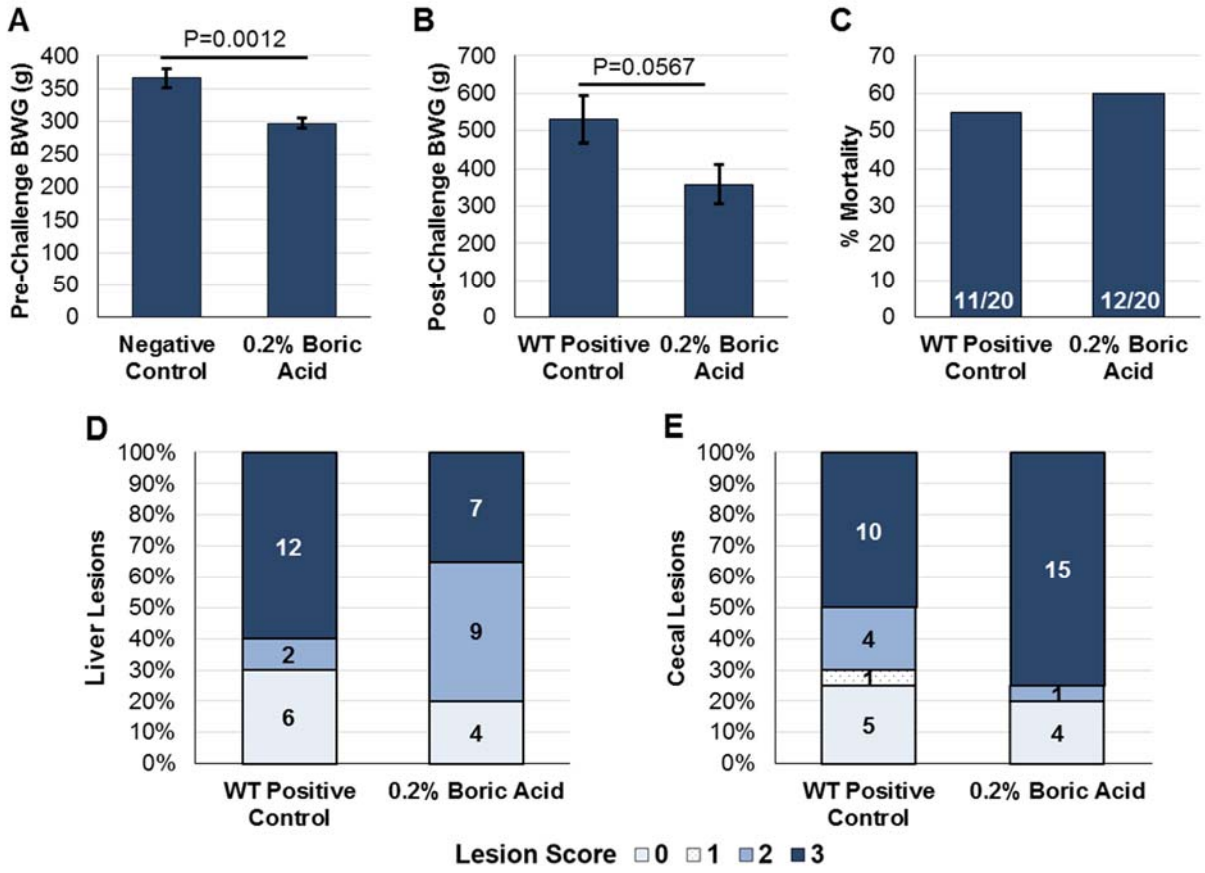

Figure 1. BWG at **A)** Pre-challenge from d0-21 and **B)** post-challenge from d21-35. BWG data expressed as mean ± SEM and were analyzed using Student's t-test in JMP Pro 14. **C)** Percentage of mortalities associated with histomoniasis post-challenge. No difference was detected when analyzed with chi square test. Cumulative lesion scores associated with histomoniasis beginning from d10-d14 post-challenge for **D)** liver and **E)** cecae. Lesions score of "0" indicative of a healthy bird with no disease whereas a score of "3" indicates classically severe histomoniasis. No differences between mean lesion scores were detected with SAS Proc Mixed Procedure for either liver or cecal lesions. Numbers within columns indicate the total poults per evaluated lesion score.

V. DATA CHAPTER 3

Evaluation of a candidate live-attenuated histomoniasis vaccine

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ABSTRACT

No vaccines are currently available to alleviate histomoniasis, a protozoal disease primarily affecting turkeys. Three experiments evaluated a live-attenuated vaccine candidate (VC) *H. meleagridis*. At selected dosages and time-points, turkeys (n=~40/group) were vaccinated via different routes (oral vs. cloacal) and subsequently challenged with a virulent wild-type (WT) *H. meleagridis*.

Experiment 1 included Negative Control and intracloacally-administered VC or WT on d14 ($2x10^5$ *Histomonas* cells/poult). On d29, a subset of Negative Control and all VC were WT-challenged. On d11 post-challenge, liver and cecal lesions were tabulated. Mortalities and lesions were significantly lower ($p \leq 0.05$) in the VC compared to the WT Positive Control.

Experiment 2 included Negative Control, VC Oral (doses: $2 x10^3$ or $2x10^4$), VC Cloacal (doses: $2x10^3$, $2x10^4$, or $2x10^5$), or WT Cloacal Positive Control ($2x10^5$). On day-of-hatch, vaccinations were administered pre-feeding via their respective routes and doses. On d21, VC groups were challenged intracloacally with $2x10^5$ WT. Two challenged-only control groups were introduced on d21, receiving the $2x10^5$ WT either cloacally or orally for the first time. Lesions were evaluated d14 post-challenge. Mortalities and lesions were higher in all groups as compared to the orally-administered WT. No differences in lesions were observed between the VC groups and the Cloacal WT Positive Control.

Experiment 3 included Negative Control, VC Cloacal (d0 or d14), VC Oral (d0), or WT Cloacal Positive Control (d0, d14, or d28). A dose of $2x10^5$ was used for both the VC and WT challenge. On d28, all treatments were challenged intracloacally with WT, and lesions evaluated d14 post-challenge. Mortalities and liver lesions were lower in the d14 VC Cloacal as compared

to WT Cloacal Positive Control. Cecal lesions were not different in any VC treatment group as compared to the WT Cloacal Positive Control.

Although histomoniasis was not completely prevented, d14 intracloacal administration of the VC reduced of lesions and mortalities in experiments 1 and 3. Taken together, these data encourage further research with the possibility that an attenuated strain could be efficacious for lessening the impact of histomoniasis in turkeys.

Key Words: blackhead, histomoniasis, *Histomonas meleagridis*, turkey, vaccination

INTRODUCTION

Histomoniasis, also commonly known as blackhead, is an important protozoal disease pertaining primarily to turkeys from the etiological origin of *Histomonas meleagridis* (Clarkson, 1963; Hess and McDougald, 2013). Flock mortality due to histomoniasis can approach 80-100%, indicating the substantial economic importance of this affliction (Callait et al., 2002; McDougald, 2005; Hess and McDougald, 2013). Direct contact between infected poultry or fecal droppings results in rapid transmission and can occur without an intermediate host or vector if transmission occurs before environmental degradation of the histomonad (Sentíes-Cué et al., 2009). The primary method of transmission is considered to be through cloacal drinking that can quickly transfer disease through the cloaca into the bursa of Fabricius or ceca via rhythmic contractions (Hu et al., 2004; McDougald and Fuller, 2005). Recently, oral administration of a clonal *in vitro* cultivated *H. meleagridis* administered to 1-day-old turkeys followed by 5h feed withdrawal resulted in mortalities associated with histomoniasis; the oral route of poultry in contact with large amounts of contaminated excreta or litter should be further considered (Liebhart and Hess, 2009).

Limited research within the past 30 years combined with the ban of therapeutic and prophylactic compounds has led to the absence of effective methods for controlling this disease (van der Heijden et al., 2005; Hess et al., 2006). Nitarsone (Histostat), the last remaining FDA-approved drug for treatment of histomoniasis, was withdrawn from the market in 2015 due to concerns over detectable levels of heavy metals retained in meat products from treated poultry (Regmi et al., 2016). *In vitro* and *in vivo* studies continue to yield variable results with no alternatives introduced to replace the recently banned effective compounds such as the

nitroimidazoles, nitrofurans, and arsenical compounds (Thøfner et al., 2012). Moreover, no vaccines are currently available to mitigate this disease.

Results from previous immunological research has been unsuccessful, contributing to doubts over the possibility of vaccine development for prevention of histomoniasis (Hu and McDougald, 2004). However, in 1963, Joyner treated turkeys suffering from acute histomoniasis with dimetridazole and reported resistance to subsequent infection in the recovered turkeys, suggesting the development of protective immunity. Cuckler (1970) reported turkeys recovered from *H. meleagridis*-infection were resistant to subsequent challenge, even with maintained presence of *H. meleagridis* within the cecae. Early studies by Tyzzer (1934) expressed the reduction of virulence within long-term *in vitro* cultivated *H. meleagridis,* although immunization results yielded conflicting success. More recent studies with chickens and turkeys have indicated reduced liver and cecal lesions following intracloacal administration of clonal *in vitro* attenuated *H. meleagridis* lines (Hess et al., 2008; Liebhart et al., 2013). Stable attenuation with no reversion to virulence was demonstrated with a *H. meleagridis* that was passaged 295 times *in vitro* and subsequently serially passaged 5 times *in vivo* in turkeys and chickens (Sulejmanovic et al., 2013). Furthermore, cross-protection against heterologous virulent isolates was demonstrated by vaccinating with an attenuated clonal strain of *H. meleagridis* developed through prolonged *in vitro* culture methods (Sulejmanovic et al., 2016). Nguyen Pham et al. (2013) cloacally inoculated turkeys with a low-virulent *H. meleagridis* strain that was obtained via serial passage in turkeys and showed induced protection following subsequent challenge with a virulent *H. meleagridis.* Moreover, Liebhart et al. (2010) demonstrated a protective effect of an *in vitro* attenuated *H. meleagridis* administered orally to 1-day-old turkeys. Development of a vaccine against histomoniasis is encouraged by these immunological research advances.

Therefore, the objective of this study was to evaluate an *H. meleagridis* strain that was passaged *in vitro* for immune protection in an experimental histomoniasis challenge model and to further elucidate the possible routes and age for administration.

MATERIALS AND METHODS

Animal Source

Poults were tagged individually and randomly allocated to floor pens at the University of Arkansas Poultry Health Laboratory on day-of-hatch. All animal handling procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC protocol #18113 and #19032) of the University of Arkansas. A corn-soy based starter feed that met or exceeded nutrient requirements of poultry (NRC, 1994) and water were provided *ad libitum*. Early poult mortalities unrelated to histomoniasis were recorded and the altered group numbers are reported in the experiments.

Histomonas meleagridis *Isolate and Culture*

A virulent wild-type (**WT)** *H. meleagridis* was obtained and cultivated based upon previously published methods (van der Heijden et al., 2005; van der Heijden and Landman, 2007). The WT was passaged approximately 80 times and chosen for evaluation as a live-attenuated vaccine candidate (**VC**). Modified Dwyer's Media (**MDM**) was used for *H. meleagridis* cultivation (Gibco, Life Technologies Corporation, USA; Lonza Walkersville Inc.; USA), supplemented with 10% heat-inactivated horse serum (Gibco) and 1.6mg/mL white rice flour. Histomonads were incubated anaerobically at 40°C for 48-72h before 1mL was sub-cultured into 12.5mL of fresh, supplemented MDM. Growth was confirmed by observation with an inverted microscope or enumeration on a hemocytometer. For long-term preservation of *H. meleagridis*, 10% dimethylsulfoxide (OmniSolv, MilliporeSigma, USA) was added as a

cryoprotectant. The suspension was then distributed into cryogenic vials (VWR, USA) and allowed to freeze at approximately -1°C/min under controlled conditions until reaching -80°C, at which time vials were transferred to long-term storage in a liquid nitrogen tank. Approximately 6 days before needed for challenge or vaccination, the desired *H. meleagridis* aliquot was retrieved from the liquid nitrogen and cultured into fresh supplemented media. Passages occurred every 48-72h. Following incubation, viable histomonads/mL were enumerated on a hemocytomer. Within each experiment, MDM was utilized as the diluent to prepare the proper dosage concentration.

Lesion Scoring System

The lesion score system was developed and initiated during "phase 2" of experiment 1 and continued throughout the remainder of the study. Classic lesions associated with histomoniasis were evaluated on a scale of "0-3", with "3" being the most severe (**Figure 1**). The individuals determining the lesion scores were blinded to the treatment groups. All mortalities were evaluated for liver and cecal lesions. According to this scale, a liver score of: "0" presents with no detectable *H. meleagridis*-related lesions, "1" indicates detectible lesions that are not clinically relevant (not significant ongoing pathology), "2" signifies intermediate lesions suggesting significant pathology but not imminent mortality, while "3" denotes confluent or nearly confluent lesions deemed likely to be fatal. The cecae were observed and palpated from the serosal surface (the mucosa was not evaluated). According to the scale, a cecal score of: "0" indicates no detectable *H. meleagridis*-related lesions observed from serosal inspection and palpation, "1" denotes thickening (not clinically significant) of the cecae, "2" indicates clinically meaningful cecal wall thickening without cecal cores, "3" specifies classic typhlitis with thickened cecal walls, inflammation, and cecal cores.

Experiment 1

Phase 1 (d14-29). A total of 120 day-of-hatch female turkey poults were obtained from a local commercial hatchery. Groups included a non-challenged Negative Control (n=59), the Vaccine Candidate (**VC**) (n=39), and the Wild-Type (**WT**) Challenged Positive Control (n=20). On d14, the VC group was inoculated with an *in vitro* attenuated (passage ~80) *H. meleagridis* at a total dose of $2x10^5$ histomonads/poult, and the WT Positive Control group received a total dose of $2x10^5$ virulent WT histomonads/poult (**Figure 2**). Inoculations were administered intracloacally with an animal gavage needle and occurred twice with approximately 1h between each inoculation. On d27 (13d post-challenge), the WT Positive Control group from "phase 1" was euthanized to evaluate characteristic disease lesions and used for comparison against the VC-induced lesions. On d28 (d14 post-vaccination), a subset of n=5 poults was sampled from both the Negative Control and VC groups to evaluate for lesions associated with histomoniasis.

Phase 2 (d29-40). On d29 (d15 post-vaccination), all poults except the Negative Control were challenged with the WT using the previously mentioned procedure. A newly introduced d29 WT Cloacal Positive Control was created from a subset of the Negative Control group to serve as concurrent reference against the VC group. All mortalities were evaluated for characteristic liver and cecal lesions pertaining to histomoniasis. On d40 (d11 post-challenge), all remaining poults were euthanized by CO_2 inhalation and lesions were tabulated according to the method described above.

Experiment 2

A total of 280 day-of-hatch female turkey poults were obtained from a local commercial hatchery and allocated to groups. Treatments included non-challenged Negative Control (n=34), VC Oral $2x10^3$ (n=36), VC Oral $2x10^4$ (n=38), VC Cloacal $2x10^3$ (n=36), VC Cloacal $2x10^4$

(n=37), VC Cloacal $2x10^5$ (n=30), and WT Cloacal Positive Control $2x10^5$ histomonads/poult (n=36). In addition, a total of 30 day-of-hatch poults were humanely euthanized for pH measurement of the combined proventriculus-ventriculus region using pH indicator strips (Sigma-Aldrich, St. Louis, MO, USA).

Phase 1 (d0-21). On day-of-hatch, poults were vaccinated prior to feeding with selected doses of either $2x10^3$, $2x10^4$, or $2x10^5$ histomonads/poult with *in vitro* attenuated (passage ~80) *H. meleagridis* administered either orally or intracloacally (**Figure 4**). The WT Cloacal Positive Control received $2x10^5$ histomonads/poult of WT, virulent *H. meleagridis* via intracloacal administration according to the method described in experiment 1. On d15, a sample from each group was evaluated for lesions to compare the VC to the WT group, leaving a remaining subset of n=20 from each VC treatment group for the following challenge "phase 2."

Phase 2 (d21-35). A WT Oral Positive Control (n=14) and WT Cloacal Positive Control (n=20) were introduced on d21 and were created from reallocation of the Negative Control poults. On d21, all poults received intracloacal challenge with WT *H. meleagridis* at $2x10^5$ total histomonads/poult in a pair of inoculations, with the exception of the WT Oral Positive Control which received this dose orally in a single administration. On d35 (d14 post-challenge), all remaining poults were euthanized by CO_2 inhalation and lesion scores were tabulated according to the method described above.

Experiment 3

A total of 480 day-of-hatch female turkey poults were obtained from a local commercial hatchery and allocated to groups. Treatments included Negative Control (n=217), d0 VC Oral (n=53), d0 VC Cloacal (n=52), d0 WT Oral Positive Control d0 (n=60), and d0 WT Cloacal Positive Control d0 (n=60). In addition, a total of 60 day-of-hatch poults were humanely

euthanized and the pH of the combined proventriculus-ventriculus region was measured in each poult using pH indicator strips (Sigma-Aldrich, St. Louis, MO, USA).

Phase 1 (d0-14). On day-of-hatch and prior to feeding, poults received selected doses of $2x10^5$ histomonads/poult of either the WT challenge or the *in vitro* attenuated (passage ~80) VC *H. meleagridis* administered either orally or cloacally according to the method described above (**Figure 7**). On d14, all poults from the WT Oral Positive Control and WT Cloacal Positive Control were evaluated for liver and cecal lesions. A total of n=10 poults/group were likewise evaluated from the Negative Control, d0 VC Oral, and d0 VC Cloacal groups to compare to the WT lesion presence.

Phase 2 (d14-28). Treatments included Negative Control (n=108), d0 VC Oral (n=43), d0 VC Cloacal (n=42), d14 VC Cloacal (n=56), and d14 WT Cloacal Positive Control (n=43). On d14, the newly introduced WT Cloacal Positive Control and VC Cloacal group received $2x10^5$ histomonads/poult of WT or VC *H. meleagridis* strain administered intracloacally, respectively. On d28, all poults from the d14 WT Cloacal Positive Control were evaluated for liver and cecal lesions. A total of n=10 poults/group were likewise evaluated from the Negative Control and d14 VC Cloacal groups. A total of n=5 poults/group were evaluated from the d0 VC Oral and d0 VC Cloacal groups.

Phase 3 (d28-42). Treatments included Negative Control (n=53), d0 VC Oral (n=38), d0 VC Cloacal (n=37), d14 VC Cloacal (n=45), and d28 WT Cloacal Positive Control (n=45). On d28, all poults except for the Negative Control, received $2x10^5$ cells/poult of the WT *H. meleagridis* challenge via intracloacal administration. On d42, all remaining poults were evaluated for liver and cecal lesions.

Statistical Analysis

Differences in mortalities associated with histomoniasis were analyzed using the chi square test with $p \leq 0.05$ considered significant. Fisher's Exact test (2018 GraphPad Software, LLC) was performed to further examine the mortality relationship between the Cloacal VC $2x10^5$ group and the Cloacal WT Positive Control when difference was not apparent via the chi square test. Lesion score data were analyzed using the Proc Mixed Procedure in SAS 9.4 software with significance between mean lesion score values denoted at p-values of ≤ 0.05, ≤ 0.005, or ≤ 0.0005 in comparison to the WT Positive Control.

RESULTS

Experiment 1

Phase 1 (d14-28). No mortalities associated with histomoniasis occurred in the Negative Control or VC, whereas the WT Positive Control reached 30% mortality before euthanasia on 13d post-challenge (d27) (**Figure 3A**). No indications of histomoniasis were observed in the Negative Controls at any time. Cumulative lesions from mortalities and scheduled termination date of the WT Positive Control revealed 75% liver and 80% cecal lesions characteristic of histomoniasis. On d28 (d14 post-vaccination), among the n=5 VC subset examined, one VC poult exhibited normal liver and cecae under gross examination, whereas two poults had presented with normal livers but relatively normal cecae with the exception of small, button-like lesions. One poult had target-like liver lesions with the cecae feeling hard, thickened, and exhibiting larger bumps and scalloping. The fifth VC poult exhibited paling edges to the liver, narrow and thin margins, and was possibly beginning to develop liver lesions. In addition, the cecae were large, with the presence of thickened walls and scalloping.

Phase 2 (d29-40). Mortalities relating to histomoniasis within the Negative Control, VC, and WT Positive Control were 0.00, 2.94, and 22.2%, respectively (**Figure 3B**). The Negative Control and VC mortalities were different ($p \leq 0.05$) as compared to the WT Positive Control. The VC displayed lower liver ($p \leq 0.0005$) and cecal ($p \leq 0.005$) lesions than the WT Positive Control (**Figures 3C and 3D**). No lesions or mortalities associated with histomoniasis were observed in the Negative Control.

Experiment 2

Phase 1 (d0-21). A mean pH of 4.4 was determined from the proventriculus-ventriculus region from the day-of-hatch poults. Mortalities related to histomoniasis in the Negative Control, VC Oral $2x10^3$, VC Oral $2x10^4$, VC Cloacal $2x10^3$, VC Cloacal $2x10^4$, VC Cloacal $2x10^5$, and WT Cloacal Positive Control were 0.00, 2.78, 2.63, 0.00, 5.41, 3.33, and 22.2%, respectively (**Figure 5A**). Mortalities in the Negative Control and all VC groups were lower ($p \leq 0.05$) than the WT Cloacal Positive Control. Liver and cecal lesions were lower ($p \leq 0.0005$) in all VC Oral and VC Cloacal groups as compared to the WT Cloacal Positive Control (**Figures 5B and 5C**).

Phase 2 (d21-35). Mortalities related to histomoniasis in the WT Oral Positive Control, VC Oral $2x10^3$, VC Oral $2x10^4$, VC Cloacal $2x10^3$, VC Cloacal $2x10^4$, VC Cloacal $2x10^5$, and WT Cloacal Positive Control were 0.00, 50.0, 50.0, 60.0, 55.0, 35.0, and 55.0%, respectively (**Figure 6A**). The WT Oral Positive Control had lower ($p \leq 0.05$) mortalities as compared to the WT Cloacal Positive Control. No differences in mortalities were found between any of the VC treatments as compared to the WT Cloacal Positive Control when evaluated with a chi square test. To further elucidate any possible difference between the VC Cloacal $2x10^5$ group as compared to the WT Cloacal Positive Control, a Fisher's Exact test was computed, resulting in $p=0.3406$. The WT Oral Positive Control resulted in lower ($p \leq 0.0005$) liver and cecal lesions as

compared to the WT Cloacal Positive Control (**Figures 6B and 6C**). The WT Oral Positive Control received only lesion scores of "0", indicating no detectable lesions associated with histomoniasis. No differences in liver or cecal lesions were found between any of the VC groups as compared to the WT Cloacal Positive Control. The p-values for the VC Cloacal $2x10^5$ as compared to the WT Cloacal Positive Control for liver and cecal lesions were p=0.1610 and p=0.6793, respectively.

Experiment 3

Phase 1 (d0-14). A mean pH of 5.0 was determined from the proventriculus-ventriculus region from the day-of-hatch poults. No mortalities associated with histomoniasis occurred in the Negative Control, VC Oral, or VC Cloacal groups (**Figure 8A**). The d0 WT Oral Positive Control and d0 WT Cloacal Positive Control resulted in 16.7 and 15% mortalities by d14 post-challenge, respectively. The d0 WT Oral Positive Control was not different in liver or cecal lesions (p=0.0908 and p=0.2360, respectively) as compared to the d0 WT Cloacal Positive Control (**Figures 8B and 8C**). The d0 VC Oral and d0 VC Cloacal were lower in liver ($p \leq 0.005$) lesions and cecal ($p \leq 0.0005$) lesions as compared to the d0 WT Cloacal Positive Control. Negative Control was lower ($p \leq 0.0005$) in liver and cecal lesions as compared to the d0 WT Cloacal Positive Control.

Phase 2 (d14-28). No mortalities associated with histomoniasis occurred in the Negative Control, d0 VC Oral, d0 VC Cloacal, or d14 VC Cloacal groups by 28-days-of-age. The d14 introduced WT Cloacal Positive Control reached 48.8% mortality by d14 post-challenge (**Figure 9A**). The d0 VC Oral, d0 VC Cloacal, d14 VC Cloacal, and Negative Control were lower ($p \leq 0.05$) in liver and cecal lesions as compared to the d14 WT Cloacal Positive Control (**Figures 9B and 9C**).

Phase 3 (d28-42). No mortalities or lesions associated with histomoniasis occurred in the Negative Control. Mortalities in the d0 VC Oral, d0 VC Cloacal, d14 VC Cloacal, and d28 WT Cloacal Positive Control reached 44.7, 32.4, 22.2, and 42.2%, respectively (**Figure 10A**). The d14 VC Cloacal group resulted in lower ($p \leq 0.05$) mortalities than the d28 WT Cloacal Positive Control. Liver and cecal lesions were different ($p \leq 0.0005$) between the Negative Control and d28 WT Cloacal Positive Control. The d14 VC Cloacal group exhibited lower ($p \leq 0.0005$) cumulative liver lesions than the d28 WT Cloacal Positive Control (**Figure 10B**). Although not statistically significant ($p=0.0899$), the d14 VC Cloacal group somewhat reduced cecal lesions as compared to the d28 WT Cloacal Positive Control (**Figure 10C**). No differences were detected in liver or cecal lesions between any of the other VC groups as compared to the d28 WT Cloacal Positive Control.

DISCUSSION

The VC lessened the severity of histomoniasis when administered at a total dosage of $2x10^5$ histomonads/turkey on d14 in experiment 1. The decreased mortalities following WT challenge and the lack of vaccine-related mortalities suggested that this passage strain of *H. meleagridis* might be attenuated enough to initiate immune response within the turkey without resulting in lethality due to the VC. Although disease was not completely prevented, the lowered lesions and mortalities as compared to the WT Cloacal Positive Control suggest that some protection was offered via the VC. Within all of the experiments, the Negative Controls never exhibited mortalities or lesions associated with histomoniasis, further confirming that disease can be prevented by management and absence of exposure.

In experiment 2, low vaccine-related mortalities associated with histomoniasis occurred in the oral (doses of $2x10^3$ and $2x10^4$) and cloacal (doses of $2x10^3$ and $2x10^5$) VC which could

potentially be explained since the *H. meleagridis* VC is not a clonal population. Variation may be occurring each time the cells are propagated, with greater or fewer virulent cells present at various concentrations depending upon the passage. During phase 1 of experiment 2, lesion presence was lower in the subsets of VC turkeys regardless of dose or route when compared to the WT Cloacal Positive Control, suggesting that the VC *H. meleagridis* was still capable of invading the tissues without causing characteristic rampant lesions. Although prolonged *in vitro* passaging of *H. meleagridis* has been reported to decrease vaccination efficacy against pathogenic strains, stable attenuation occurred in other studies without reversion to virulence (Lund and Chute, 1967; Sulejmanovic et al., 2013).

Oral transfer of *H. meleagridis* should not be overlooked as previous studies with chickens have demonstrated that feed deprivation and an alkaline pH prior to oral challenge were required in order to develop lesions characteristic with histomoniasis (Cuckler, 1970). Interestingly, WT oral challenge on d21 resulted in no mortalities or lesions associated with histomoniasis, consistent with the prevailing thoughts that *H. meleagridis* cannot survive the low pH within the proventriculus and ventriculus typically present following feed consumption. Conversely, in experiment 3, turkeys that were WT challenged orally on d0 were equally susceptible to *H. meleagridis* infection with no differences noted in mortalities or lesions as compared to the WT Cloacal Positive Control. Following feed ingestion, the average pH in the proventriculus/ventriculus has been reported as 3.5 in broiler chickens with variability between 1.9 and 4.5 (Svihus, 2011). The susceptibility of the poults on day-of-hatch pre-feeding could likely be explained by the higher pH (measured as 4.4 and 5.0 in experiments 2 and 3, respectively) within the proventriculus/ventriculus region, indicating a more basic environment for which the protozoa could potentially survive until parasitizing the cecae. If repeated in future

studies, pH should be measured in a subset of turkeys post-feeding to compare to pre-feeding measurements. Unexpectedly, the subset of the d0 VC Oral (dose $2x10^5$) in experiment 3 exhibited some mild to severe lesions characteristic with histomoniasis when evaluated on d28 post-vaccine, indicating the ability of the VC *H. meleagridis* to invade tissue although no mortalities related to the disease occurred during this timeframe.

Some turkeys within the WT Cloacal Positive Control group in each experiment exhibited lesion scores of "0", indicating no abnormality or pathology of histomoniasis. The absence of lesions within a subset of the WT Cloacal Positive Control could potentially be explained in that some turkeys may be less susceptible to challenge, cecal retrograde of the parasite inoculum could vary between turkeys, or some turkeys may excrete the inoculum before cloacal uptake occurs. Moreover, the absence of clonal culture could contribute to prevalence of certain cell populations with greater or lower degrees of virulence. Although passages of the WT *H. meleagridis* are minimized to prevent *in vitro* attenuation in culture, a chance of virulence or population changes are present with each propagation. To reduce this concern, both the WT and VC should be single-cell cloned and evaluated to ensure the same genetic population is being evaluated in subsequent experiments.

Liebhart et al. (2010) demonstrated protectiveness of an *in vitro* attenuated *H. meleagridis* administered to 1-day-old turkeys. However, under the conditions of these experiments, the cloacal route appears to be the more efficacious route as compared to the oral route for administration, especially at an older age. Administration of the VC *H. meleagridis* intracloacally on d14 resulted in lower mortalities and lesions in both experiments 1 and 3, suggesting that this might be an efficacious alternative for the prevention of histomoniasis. Intracloacal inoculation with attenuated *H. meleagridis* has shown induced protection as well as

cross-protective capability against virulent isolates (Nguyen Pham et al., 2013; Sulejmanovic et al., 2016). Further research should be conducted to elucidate the most efficacious route and dosage of the VC *H. meleagridis* as well as the proper age for administration. It should be noted that other passage isolates of *H. meleagridis* have been evaluated by other research groups and shown to induce protection against subsequent challenge, as outlined above. Additionally, if our selected VC is efficacious at alleviating histomoniasis and is successfully single-cell cloned, we will need to address a major limiting factor to larger scale production of the live-attenuated *H. meleagridis* strain, specifically more efficient methods to feasibly propagate cells to meet commercial production needs. The current cell culture methods seem unfeasible for mass production, as the cells grow at varied rates and the media is relatively costly. Moreover, for introduction to the industry, the intracloacal route would not be the most practical technique for large-scale application. The d0 administration would be preferable for industry application for incorporation within the hatchery. Presumably, oral route at day-of-hatch seems the most efficient, if possible. However, if the cloacal route proves to be the only effective administration to induce a robust immune response, then potentially an alternative method could be developed for cloacal application during the beak trimming, toenail removal, or sex determination procedure at the hatchery. Certainly, a main consideration is the identification of an effective prophylaxis for this life-threatening disease of poultry. The current lack of any approved prophylactic measures against histomoniasis suggests that any contribution to the mitigation of this disease is substantial to improving animal food production and reducing this emerging problem.

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FIGURES

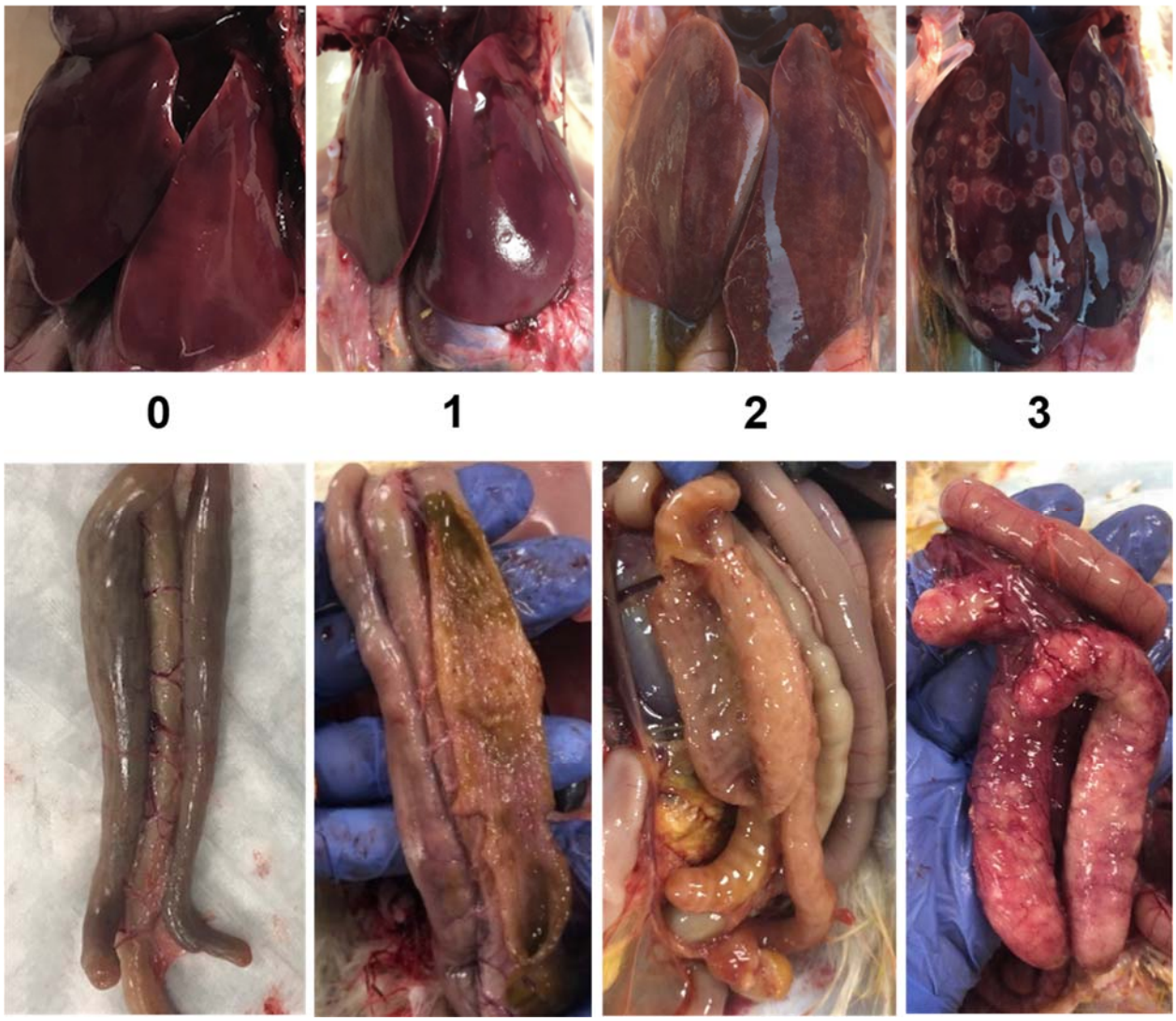

Figure 1. Histomoniasis lesion scoring system developed at the University of Arkansas Poultry Health Laboratory. Classic lesions associated with histomoniasis for liver and cecae were evaluated on a scale of "0"-"3." A score of "0" indicates no detectible lesions; "1" indicates lesions not clinically relevant; "2" signifies intermediate lesions suggesting significant pathology but not imminent mortality; "3" denotes confluent or nearly confluent lesions deemed likely to be fatal.

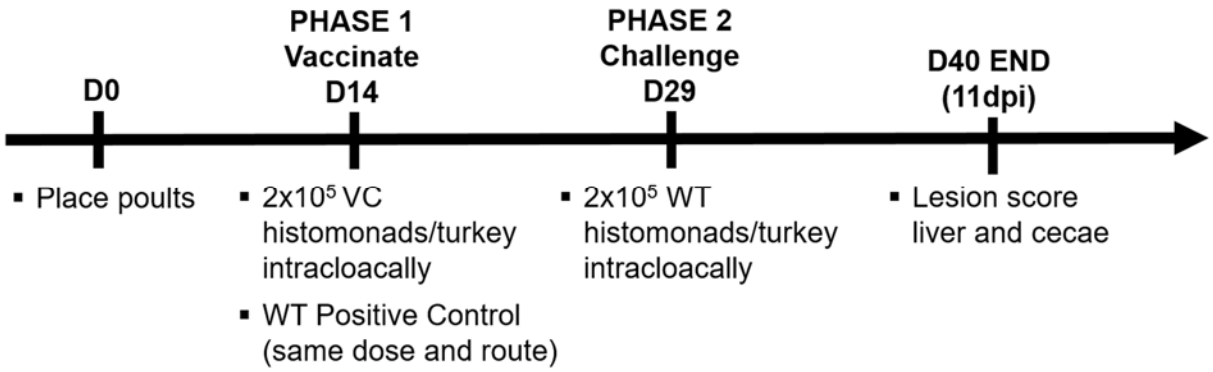

Figure 2. Timeline for Experiment 1. VC=Vaccine Candidate; WT=Wild-Type.

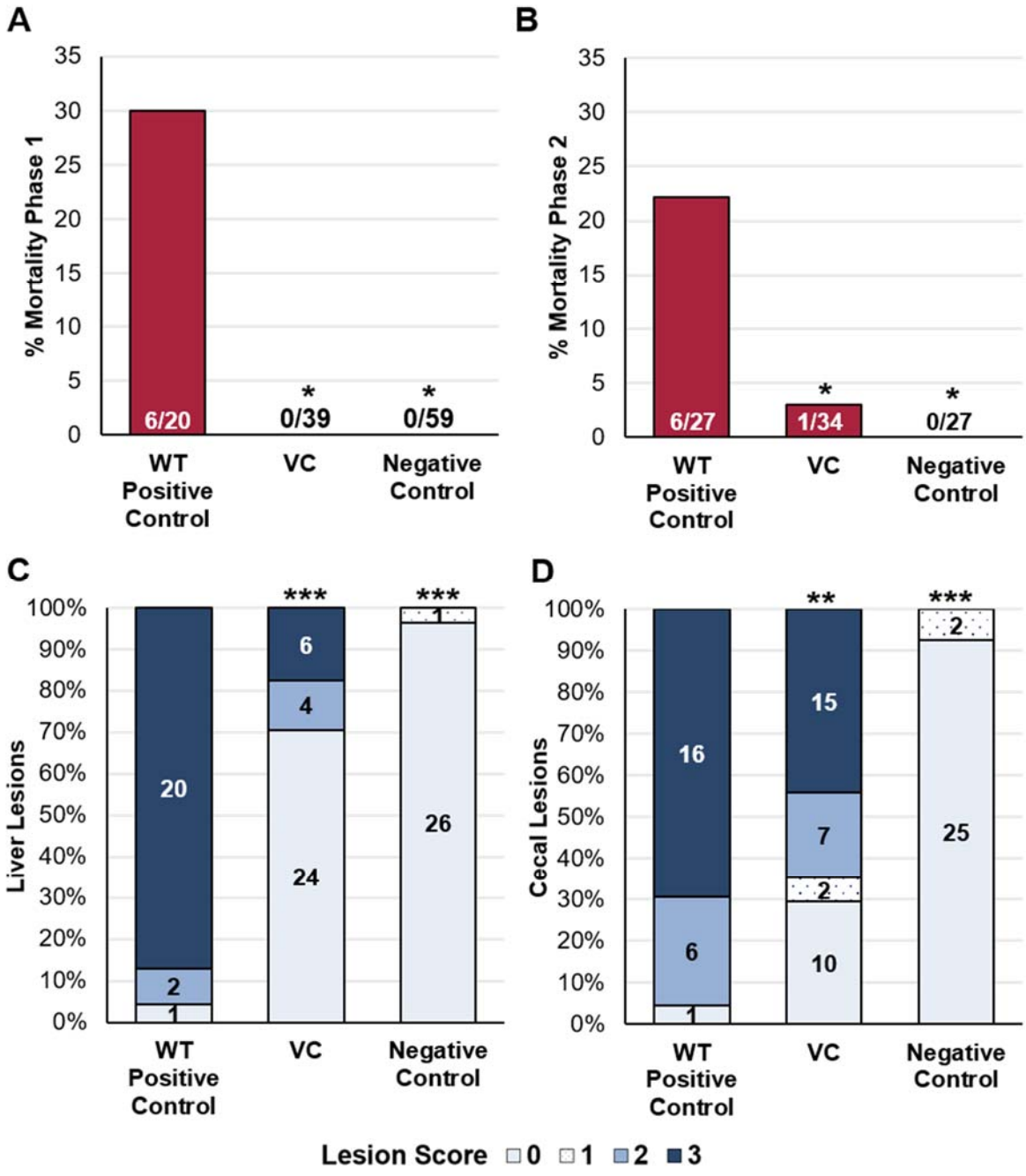

Figure 3. Experiment 1 cumulative percentage mortalities associated with histomoniasis at **A)** Phase 1 (d14-29) and **B)** Phase 2 (d29-40). Lesion scores on 11d post-challenge for **C)** liver and **D)** cecae. Statistical difference detected by the SAS Proc Mixed Procedure between mean lesion scores as compared to the Wild-Type (WT) Positive Control group is indicated by "*" for $p \leq 0.05$, "**" for $p \leq 0.005$, and "***" for $p \leq 0.0005$. Numbers within columns indicate the total poults per evaluated lesion score. VC=Vaccine Candidate.

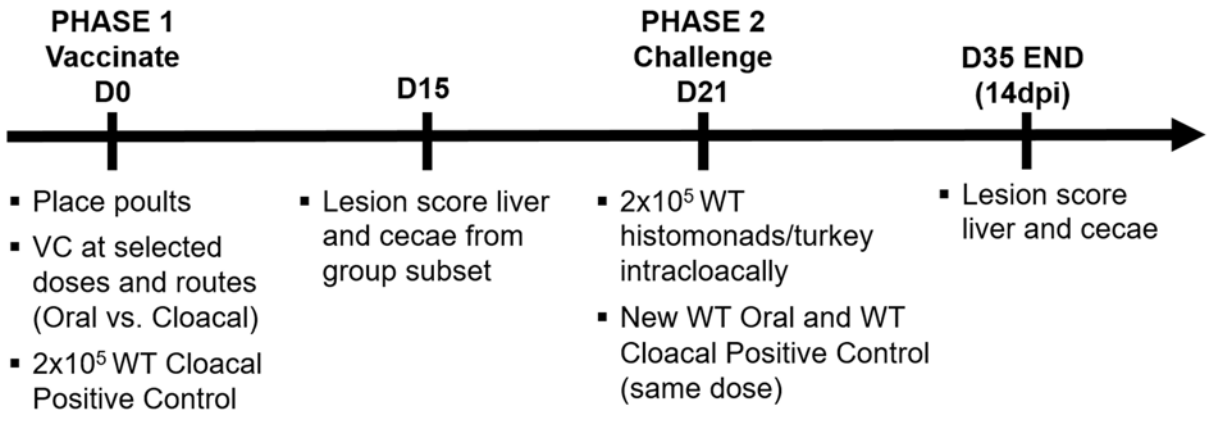

Figure 4. Experiment 2 timeline. VC=Vaccine Candidate; WT=Wild-Type.

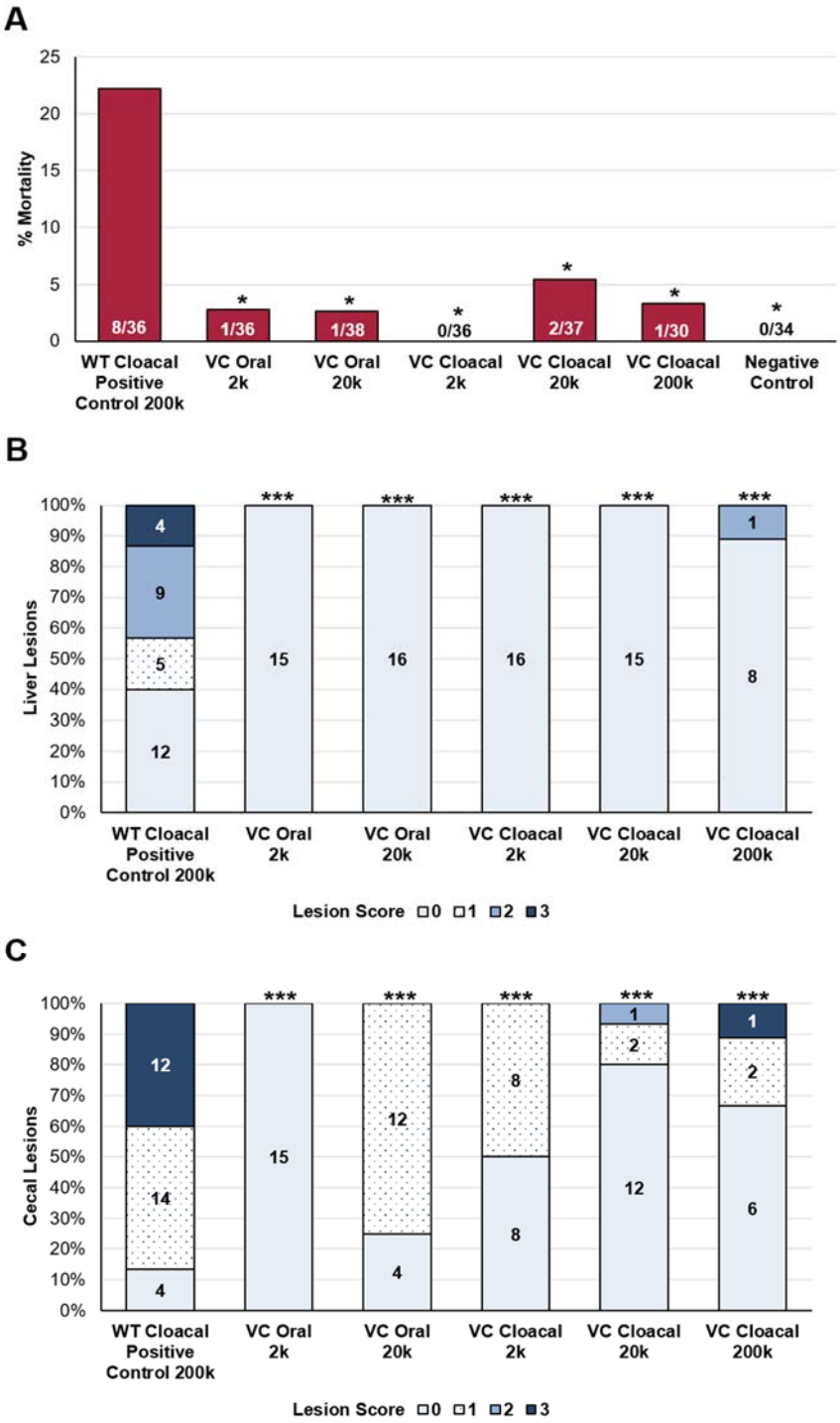

Figure 5. Experiment 2 response during Phase 1 (d0-21) for **A)** cumulative percentage of mortalities associated with histomoniasis, lesion scores of **B)** liver and **C)** cecae at 15d post-administration of Vaccine Candidate (VC) or Wild-Type (WT) *Histomonas meleagridis*. Statistical difference detected by the SAS Proc Mixed Procedure between mean lesion scores as compared to the Wild-Type (WT) Positive Control group is indicated by "*" for $p \leq 0.05$, "**" for $p \leq 0.005$, and "***" for $p \leq 0.0005$. Numbers within columns indicate the total poults per evaluated lesion score. VC=Vaccine Candidate.

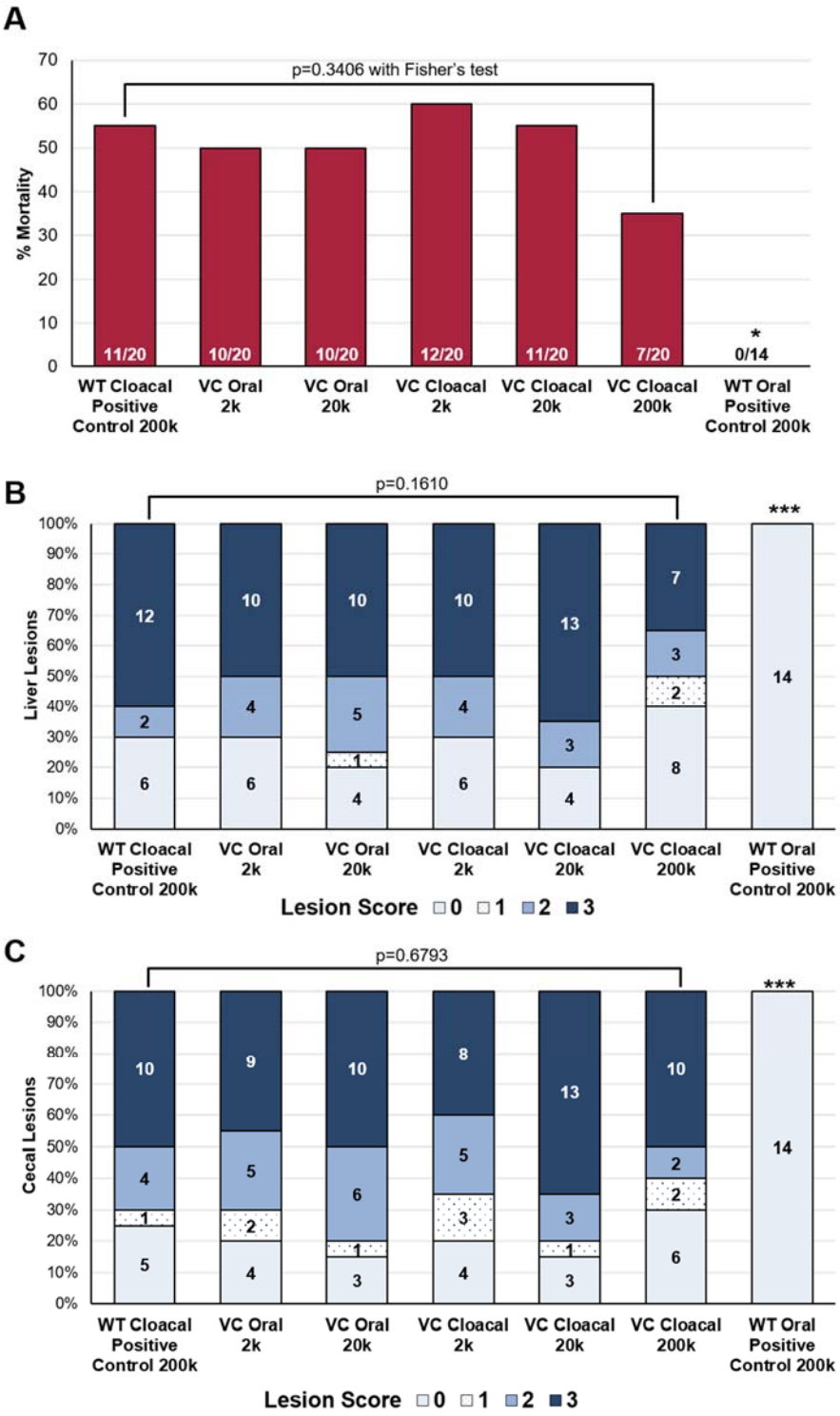

Figure 6. Experiment 2 response during Phase 2 (d21-35) for **A**) cumulative percentage of mortalities associated with histomoniasis, lesion scores of **B)** liver and **C)** cecae beginning from d9-14 post-WT challenge. Statistical difference detected by the SAS Proc Mixed Procedure between mean lesion scores as compared to the Wild-Type (WT) Positive Control group is indicated by "*" for $p \leq 0.05$, "**" for $p \leq 0.005$, and "***" for $p \leq 0.0005$. Numbers within columns indicate the total poults per evaluated lesion score. VC=Vaccine Candidate.

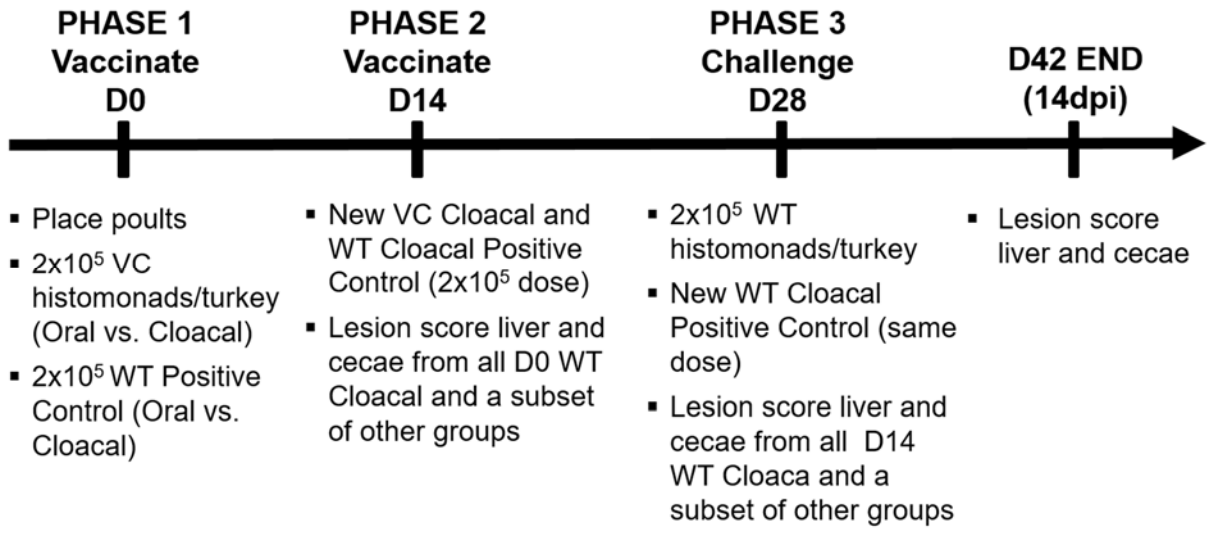

Figure 7. Experiment 3 timeline. VC=Vaccine Candidate; WT=Wild-Type.

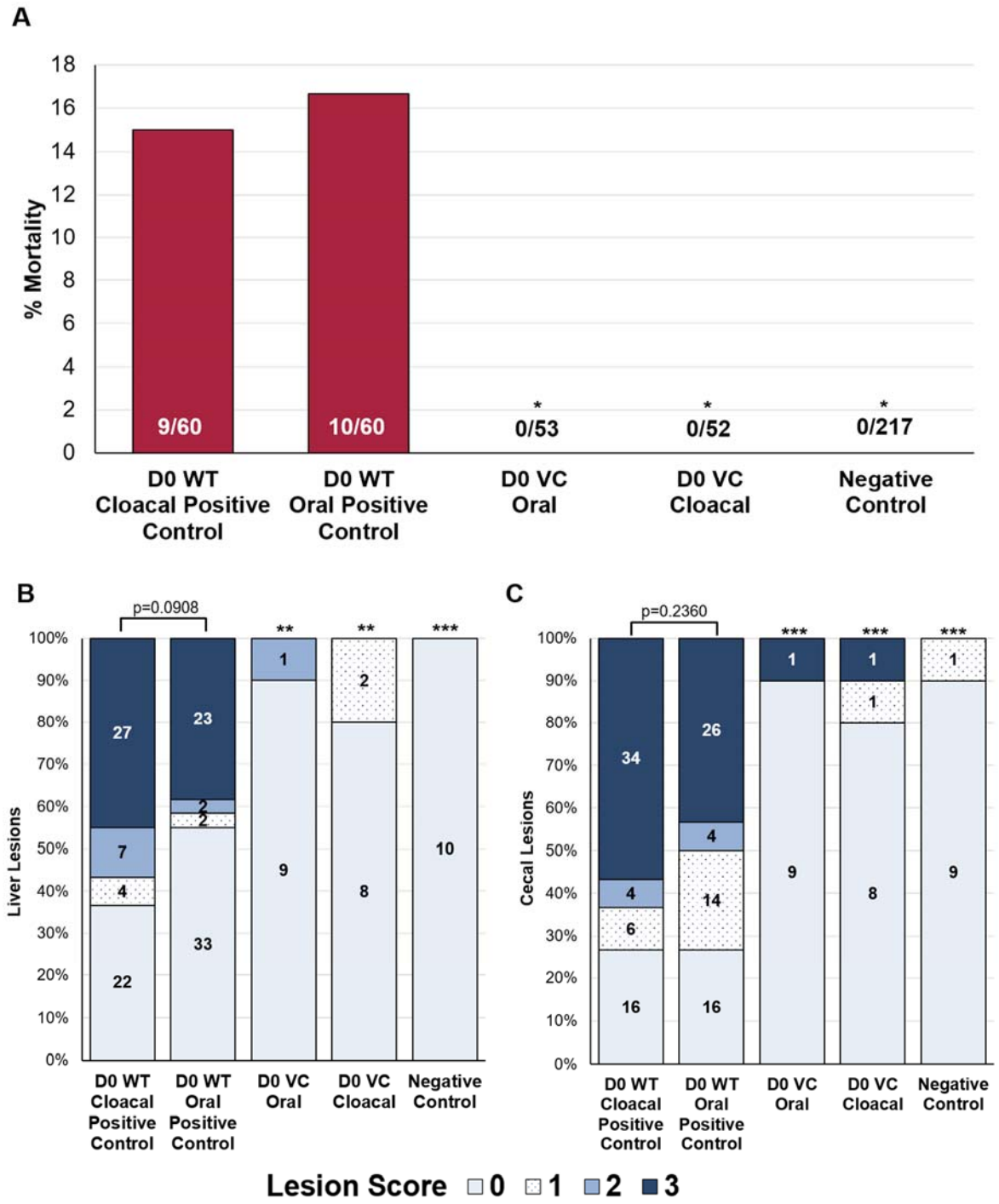

Figure 8. Experiment 3 response during Phase 1 (d0-14) for **A)** cumulative percentage of mortalities associated with histomoniasis, lesions scores of **B)** liver and **C)** cecae on d14 post-administration of Vaccine Candidate (VC) or Wild-Type (WT) *Histomonas meleagridis*. Statistical difference detected by the SAS Proc Mixed Procedure between mean lesion scores as compared to the Wild-Type (WT) Positive Control group is indicated by "*" for $p \leq 0.05$, "**" for $p \leq 0.005$, and "***" for $p \leq 0.0005$. Numbers within columns indicate the total poults per evaluated lesion score. VC=Vaccine Candidate.

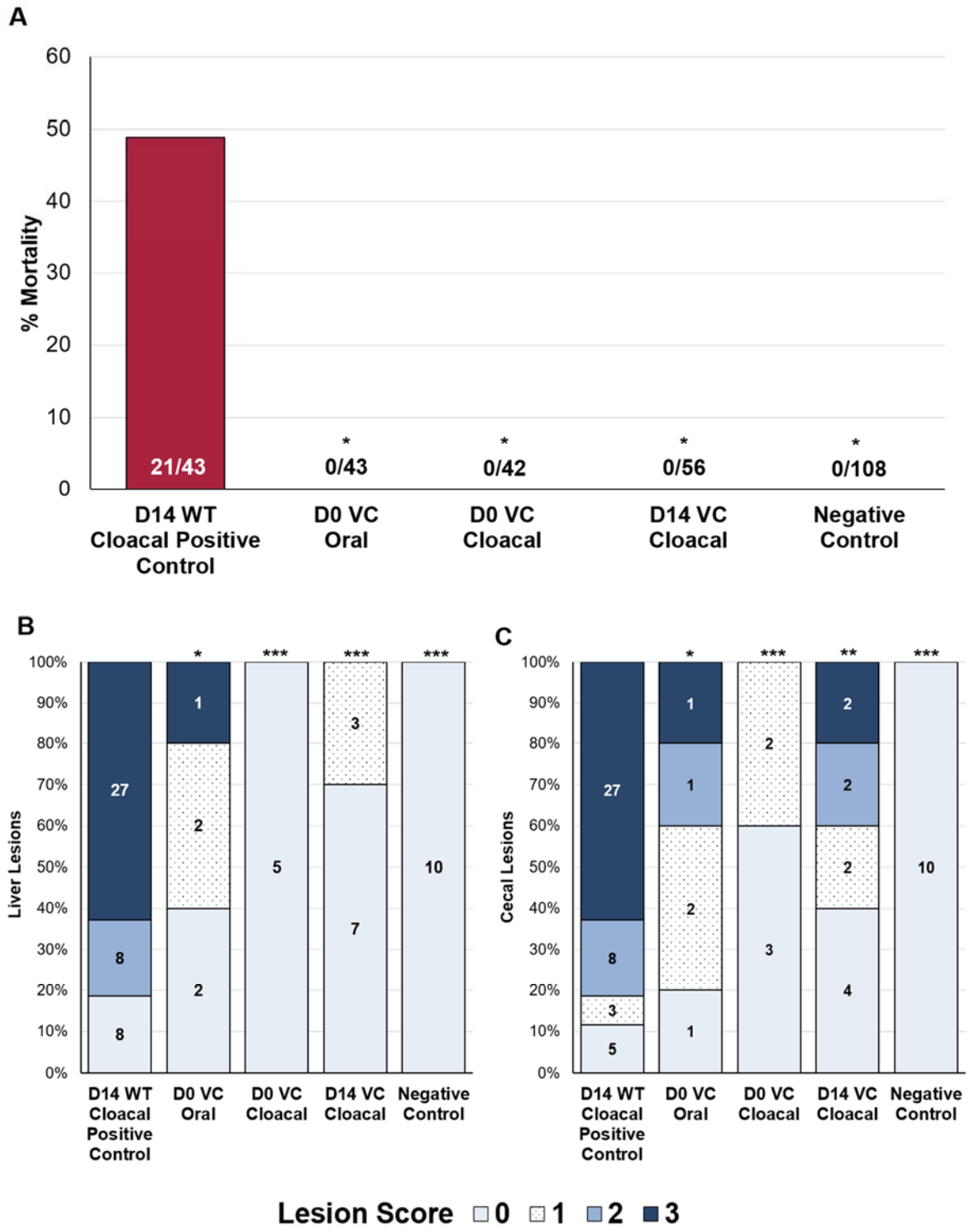

Figure 9. Experiment 3 response during Phase 2 (d14-28) for **A)** cumulative percentage of mortalities associated with histomoniasis, lesion scores of **B)** liver and **C)** cecae. Statistical difference detected by the SAS Proc Mixed Procedure between mean lesion scores as compared to the Wild-Type (WT) Positive Control group is indicated by "*" for $p \leq 0.05$, "**" for $p \leq 0.005$, and "***" for $p \leq 0.0005$. Numbers within columns indicate the total poults per evaluated lesion score. VC=Vaccine Candidate.

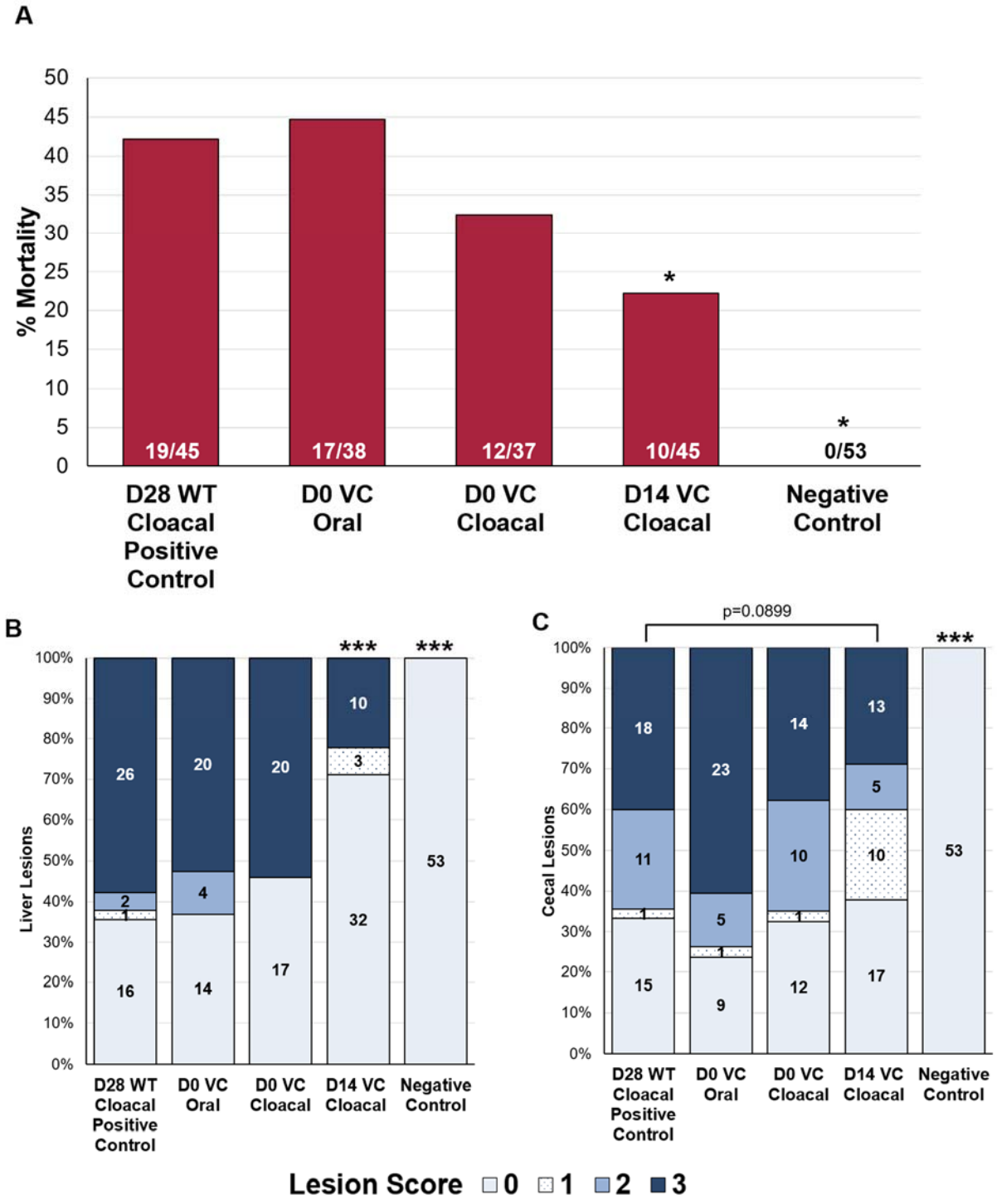

Figure 10. Experiment 3 response during Phase 3 (d28-42) for **A)** cumulative percentage of mortalities associated with histomoniasis, lesion scores of **B)** liver and **C)** cecae. Statistical difference detected by the SAS Proc Mixed Procedure between mean lesion scores as compared to the Wild-Type (WT) Positive Control group is indicated by "*" for $p \leq 0.05$, "**" for $p \leq 0.005$, and "***" for $p \leq 0.0005$. Numbers within columns indicate the total poults per evaluated lesion score. VC=Vaccine Candidate.

VI. CONCLUSIONS

None of the dietary treatments of deoxycholic acid (**DCA**), biliogenic formulation, or boric acid reduced disease within *H. meleagridis*-challenged turkeys. Although DCA exhibited anti-histomonal properties *in vitro*, efficacy was not transferred *in vivo* under the experimental conditions of this study. These results further confirm the complicated nature of this disease as well as the importance of *in vivo* evaluation rather than reliance only upon *in vitro* methods.

Under the conditions of experiment 1 within the vaccination study, the Vaccine Candidate (**VC**) *Histomonas meleagridis* resulted in lowered disease-related mortalities and lesions as compared to the Wild-Type Cloacal Positive Control. Although histomoniasis was not completely prevented, d14 intracloacal administration of the VC lowered lesions and mortalities in experiments 1 and 3. Taken together, these data suggest the potential efficacy of the VC to protect from histomoniasis. Further research should be conducted to elucidate the most effective dosage, route, and age for administration of this VC.

APPENDIX

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Craig Coon
Date:	April 12th, 2018
Subject:	IACUC Approval
Expiration Date:	April 5th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18113:** *Development and evaluation of prophylactic treatments for Histomoniasis in turkeys*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 5th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Lesleigh Beer, and Cheryl Lester. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

18113

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Billy Hargis
Fr:	Craig Coon
Date:	October 8th, 2018
Subject:	IACUC Approval
Expiration Date:	October 4th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **19032:** *Development and evaluation of prophylactic treatments, vaccinations, and routes of administration on the prevention of Histomoniasis in turkeys*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond October 4th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Billy Hargis, Guillermo Tellez, Lesleigh Beer, Cheryl Lester, Thaina Barros, and Christine Vuong. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp